IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit: 1646
COX et al.	Examiner: Xie, Xiaozhen
Serial No.: 10/031,154	**DECLARATION OF GEORGE COX AND DANIEL DOHERTY UNDER 37 CFR 1.131**
Filed: January 14, 2002	
Atty. File No.: 4152-3-PUS	
For: "IMMUNOGLOBULIN FUSION PROTEINS"	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

We, George Cox and Daniel Doherty, each declare as follows:

1. I am a co-inventor of the above-referenced patent application and am familiar with the application.

2. This Declaration under 37 CFR §1.131 is being submitted in conjunction with a Response to an Office Action mailed on September 25, 2008, the Response being filed herewith.

3. This Declaration provides factual evidence of the conception of the invention as claimed in *at least* Claims 90-94, 96, 102, 104, 105, 130-135 and 139, at a date prior to the July 24, 1998 effective filing date of the material cited in Blumberg, et al., U.S. Patent No. 6,485,726 in support of the rejections under 35 U.S.C. § 103(a), followed by diligence in reduction to practice from a date prior to the effective filing date of Blumberg, et al. (July 24, 1998) to the date of constructive or actual reduction to practice of Claims 90-94, 96, 102, 104, 105, 130-135 and 139. All acts relied upon to establish the dates of conception, diligence and reduction to practice were carried out in the United States.

Evidence of Conception prior to July 24, 2008

As evidence of conception of the invention as claimed in Claims 90-94, 96, 102, 104, 105, 130-135 and 139, at a date prior to July 24, 1998, enclosed as Exhibit A are relevant portions of a grant application that was prepared by us and submitted at a date prior to July 24, 1998.

This grant application proposes the construction and production of Epo-Ig fusion proteins (all claims), including Epo/IgG-Fc and Epo/IgG-CH fusion proteins (Claims 90 and 139), with biological activities (EC_{50}'s) that are comparable to wild-type EPO on a molar basis, which is encompassed by all of the EC_{50} values claimed (Claims 90 and 139). The grant sections describe joining the C-terminus of EPO to the N-terminus of human IgG-Fc or IgG-CH domains using a restriction site to join the protein domains (Claims 90, 139 and dependents therefrom).

More specifically, the grant sections provided show the amino acid sequence and structural organization of human EPO and provide details for the production of the constructs using unique restriction sites to join the EPO and IgG-Fc or IgG-CH sequences. On page 11, lines 14-22 of the application, we state that the junction sequences (peptide linkers) will be the only non-natural sequences in the fusion protein, thereby teaching the use of a linker to connect the proteins (Claims 90, 92-94, and 132-135). We describe how we will amplify the genes encoding EPO and the Ig portions of the fusion protein using, *e.g.*, PCR, and clone them into mammalian cell expression vectors (Claim 104). We discuss how to express proteins in mammalian COS or CHO cells (Claim 104). We state that we expect the proteins to be secreted as homodimers, and we state that we will purify the proteins by affinity chromatography using protein A columns (Claims 102, 104, 105). We state that bioactivities of the proteins can be measured using the UT7/epo cell line and again, propose EPO-Ig fusions with biological activities comparable to wild-type EPO (Claims 90 and 139).

Evidence of Diligence beginning prior to July 24, 1998

(1) As evidence of diligence beginning prior to July 24, 1998, enclosed as Exhibit B are documents showing that after the filing of the grant application, and prior to July 24, 1998, we monitored the progress of the grant application. On or prior to July 24, 1998, we received communications from the granting agency including a grant priority score, a summary statement containing reviewer critiques, and a letter indicating that the agency intended to fund the grant pending resolution of the time commitment and employment issues.

(2) As evidence of diligence beginning prior to July 24, 1998, enclosed as Exhibit C are relevant portions of notebook pages that show that we were diligently performing experiments to reduce the invention as claimed in Claims 90-94, 96, 102, 104, 105, 130-135 and 139 to practice. Specifically, these notebook pages, spanning dates from June 3, 1998 to July 15, 1998 describe the planning and execution of experiments to amplify the genes encoding EPO.

Namely BB41 and BB42 oligonucleotides for PCR cloning Epo cDNA were designed and obtained, and Quickclone cDNA was obtained. PCR cloning was attempted and the first attempt was unsuccessful (notebook pages 97, 98 and 103-106).

An attempt to make RNA from human Hep 3B cells (which had been reported to express Epo under hypoxic conditions) is described on notebook pages 107-112.

An attempt to use RT-PCR to amplify Epo cDNA from human liver RNA (unsuccessfully) is shown on notebook pages 15-16.

PCR primers BB45 and BB46 for cloning Epo were ordered (page 113). A new PCR primer BB47 was also ordered. (page 113). PCR amplified of Epo cDNA from Hep 3B RNA using BB45 and BB47 oligos was successful. (pages 34-35). The Epo cDNA was cloned into pUC19 plasmid DNAs and sent to Macromolecular Resources in Fort Collins for DNA sequencing (page 34-42). Epo clone #10 had the correct DNA sequence and was called plasmid pBBT131, pUC19::Epo E10 (page 42).

(3) As further evidence of diligence prior to July 24, 1998, enclosed as Exhibit D are documents showing that we also contacted researchers regarding the UT7/Epo cell line and how to obtain it. We received an email from the researcher who isolated the cell line confirming that a second researcher had permission to send us the UT7/Epo cell line.

Evidence of Continued Diligence and Constructive and/or Actual Reduction to Practice

(1) After July 24, 1998 we were taking steps to reduce the invention as claimed in Claims 90-94, 96, 102, 104, 105, 130-135 and 139 to practice. We ordered human TF1 cell line from ATCC for testing Epo proliferation. We also ordered recombinant human Epo from R&D Systems, Inc. for testing TF-1 cell line response. Monkey COS cells were ordered from ATCC for transient transfection experiments. See Exhibit E.

(2) After July 24, 1998, we responded to the granting agency regarding the employment and time commitment, and the grant funding began, also shown in Exhibit F.

(3) Exhibit G represents an experiment completed October 1-9, 1998 that provides evidence that we were diligently performing experiments to reduce the invention as claimed in Claims 90-94, 96, 102, 104, 105, 130-135 and 139 to practice. Specifically, these notebook pages describe the planning of different EPO-Ig fusion constructs and then the initial production of constructs encoding immunoglobulin and EPO proteins that were used to produce various EPO-Ig fusion proteins. These constructs were used to produce the linkered fusion proteins. Specifically, these notebook pages show diagrams for fusion constructs of EPO and IgG_1 and IgG_4 (Fc or hinge and CH domains), as well as marked up sequences for EPO and IgG_1 and IgG_4, and oligonucleotide design, as well as experiments showing successful cloning and expression of the individual components that were used to create EPO-Ig fusion proteins as claimed.

(4) Exhibit H also represents an experiment completed on January 12-15, 1999 which provides evidence that we were diligently performing experiments to reduce the invention as claimed in Claims 90-94, 96, 102, 104, 105, 130-135 and 139 to practice. In particular, this experiment establishes that we were diligently performing experiments to reduce to practice the construction, production, and testing of biologically active EPO-Ig fusions, beginning with EPO-Ig fusions having the recited small peptide linkers consisting of serine and glycine amino acids.

The three fusion proteins are denoted pBBT 179, pBBT 180 and pBBT 181. Construct pBBT 179 is EPO-IgG_1/CH_1 (EPO-IgG_1-CH); construct pBBT 180 is EPO-IgG_1/hinge (IgG_1-Fc); and construct pBBT 181 is EPO-IgG_4/hinge (IgG_4-Fc). In these fusions, the peptide linker was 7 amino acids in length (Claims 90, 92, 133, 134, 135, 136, 138 and 139) and consisted of glycine and serine residues (Claims 90, 92, 133, 134, 135, 136, 138 and 139). The Ig portion of the fusion protein was either IgG1-Fc, IgG4-Fc or IgG1-CH, demonstrating that we had produced fusion proteins using two different Ig isotypes and both Fc and CH fusions. Prior to the experiment described in Exhibit H below, the three fusion protein nucleotide constructs were produced using recombinant techniques, and the fusion proteins were expressed by transfecting a host cell with an expression vector comprising the recombinant constructs, culturing the host cell under conditions effective to express the fusion protein, and harvesting the fusion protein expressed by the host cell (Claim 104). The expression of the fusion proteins was demonstrated and levels of expression quantitated by Western blots using anti-EPO antibodies in order to determine the concentrations of the proteins prior to putting them into the bioassay described in Exhibit H below (data not shown in this Declaration).

The EC_{50}'s (as defined in the present application, the concentration of protein required for half-maximal stimulation) of each of the three EPO-Ig fusion proteins was measured in the UT7/epo cell proliferation bioassay, shown in the notebook pages of Exhibit H. The first page of the Exhibit shows the three fusion proteins tested as discussed above (BBT 179, BBT 180, BBT 181), with plasmid pCDNA3.1 serving as a negative control and a wild type human EPO protein purchased from R & D Systems, Inc. serving as a positive control. Plasmids BBT179, BBT180 BBT181 and pcDNA3.1 were used to transfect COS cells and the conditioned media containing the fusion proteins was harvested several days later. Serial 10-fold dilutions of the conditioned media were prepared and assayed in the UT7/epo cell proliferation assay. The serial dilutions were called tubes 1-6. Estimated concentrations of the EPO-IgG fusion proteins in the assay were: tube 1, 0.0005 ng/mL; tube 2, 0.005 ng/mL; tube 3, 0.05 ng/mL; tube 4, 0.5 ng/mL; tube 5, 5 ng/mL; and tube 6, 50 ng/mL. Six serial dilutions of the EPO control protein were prepared as well. Concentrations of the EPO standard in the assay were: tube 1, 0.0004 ng/mL; tube 2, 0.004 ng/mL; tube 3, 0.04 ng/mL; tube 4, 0.4 ng/mL; tube 5, 4 ng/mL and tube 6, 40 ng/mL. The second page of Exhibit H shows the experimental set-up for each of the three test plates (called plates A, B and C). Each of the serial dilutions was assayed in triplicate. Additional control wells contained no cells (called "no cells") or just media but no Epo or Epo-Ig (called "0"). The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase in absorbance values, which is proportional to cell number. In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. The results shown on the third-sixth pages of the Exhibit provide the raw data and graphs of the activity of the three EPO-Ig fusion proteins as compared to rEPO and a negative control. The graphs plot absorbance of the wells on the Y-axis versus the dilution tube on the X-axis (the percent of the COS cell supernatant (% sup) in the dilution tubes for the fusion proteins or the number of EPO units/mL (1 unit = 8 ng/mL) in the dilution tubes also is plotted on the X-axis under the appropriate dilution tube). The dilution tube closest to the EC_{50} for each fusion protein was tube 4, which contained an estimated 0.5 ng/mL of the fusion protein. The serial dilution tube closest to the EC_{50} for the EPO control protein also was tube 4, which contained an estimated 0.4 ng/mL of EPO. Thus, the EC_{50}s of the fusion proteins were within the scope of less than 4 ng/ml, and comparable to (within at least 4 fold) activity of wild type EPO on a molar basis, which also

represents an EC_{50} of less than 1000 ng/ml and less than 10 ng/ml (Claims 96, 134, 135, and 136).

(5) Between January 15, 1999 and July 13, 1999, we continued to design constructs and perform experiments to produce and test additional EPO-Ig fusion proteins as claimed in Claims 67, 68, 77, 78, 80-87, 89-94, 96, 102, 104, 105, and 125-138, and we worked with patent counsel to constructively reduce the invention to practice by the preparation and filing of U.S. Provisional Application No. 60/143,458, filed July 13, 1999, which is the priority document for the present application. The following Exhibits describe activities and representative experiments that pertained to the constructive and actual reduction to practice of the invention during this time period.

Exhibit I contains notebook pages dated from February 9-17, 1999, showing the results of an experiment that began on January 21, 1999 (see reference to 1/21/99 transfection), showing the larger scale transfection of host cells with the EPO-Ig fusion constructs described in the Exhibits above and the purification of the fusion proteins (purification of EPO-IgG_4/hinge (Fc) or pBBT 181, EPO-IgG_1/CH or pBBT 179, and EPO-IgG_1/hinge (Fc) or pBBT 180 is shown).

(6) Exhibit J contains a notebook page date February 26, 1999, showing the design and beginning of the construction of a recombinant construct encoding a different EPO-Ig fusion protein, which was an EPO joined at its carboxy-terminus to IgG_4-CH (an immunoglobulin domain that does not contain a variable region) using a linker comprised of serine and glycine residues. Note the diagram illustrating the construct plan.

(7) Exhibit K contains notebook pages showing an experiment beginning on March 23, 1999, and ending on April 28, 1999, describing the COS cell transfection, expression and purification of the new EPO-Ig fusion protein (EPO-IgG_4/CH (pBBT 185)) described in Exhibit J above.

(8) Exhibit L provides relevant portions of a grant application completed and filed on April 14, 1999, which includes particular experimental details regarding the construction and production of EPO-Ig fusion proteins having 2 and 4 amino acid linkers, and EPO-Ig fusions having no intervening linker. This grant also includes much of the material described in the grant sections of Exhibit A and presents the data shown in Exhibit H and related experiments and specifically, describes the detailed construction, production and testing of EPO-IgG-Fc and EPO-

Ig-CH fusions with a peptide linker. This document also describes specific methods for separating monomers from dimers.

(9) Exhibit M contains notebook pages showing the results on June 8-9, 1999, of an experiment that began April 30, 1999, showing the purification of a large scale EPO-IgG_1-Fc fusion protein (pBBT 180) transfection experiment.

(10) Exhibit N contains a notebook page from an experiment performed on July 6-9, 1999, showing the bioactivity for the EPO-IgG_4-CH fusion protein (pBBT 185) and for the scaled up expression of the EPO-IgG_1-Fc fusion protein (pBBT 180). The EC_{50}s for the fusion proteins are shown as compared to wild-type recombinant EPO. The EC_{50} for the EPO control protein was 0.48 and 0.48 ng/mL in two assays; the EC_{50}s for BBT185 were 2 and 2.1 ng/mL in two assays and the EC_{50}s for BBT180 were 1.3 and 1.3 ng/mL in two assays.

(11) Exhibit O is U.S. Provisional Application Serial No. 60/143,458, filed July 13, 1999, which constructively reduces to practice the invention as claimed in Claims 90-94, 96, 102, 104, 105, and 130-135.

4. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

March 25, 2009
Date

George Cox

Date

Daniel Doherty

EXHIBIT A

***Research Plan:**

***A. Specific aims:**

* There is considerable interest on the part of patients and healthcare providers in the development of *low cost, long-acting, "user-friendly" protein therapeutics. Most protein pharmaceuticals have short *circulating half-lives in the body and must be injected daily or every other day for maximum effectiveness. *For example, growth hormone (GH) and granulocyte colony-stimulating factor (G-CSF) require daily *injections and erythropoietin (EPO) requires every other day injections for maximum effectiveness. These *recombinant proteins have proven extremely effective at treating short stature and cachexia (GH), *neutropenia (G-CSF) and anemia (EPO). For each of these proteins it is known that increasing the *circulating half-life of the protein improves the protein's in vivo performance. We propose to create *longer-acting forms of GH, G-CSF and EPO through covalent fusion of these proteins to the heavy chain *domain of human IgG1. Human IgG1 has a long serum half-life, on the order of 21 days. Fusion of *several other proteins, principally extracellular domains of cell surface receptors, to the IgG1 heavy chain *domain has resulted in increased serum half-lives for these proteins. Despite this success, IgG fusion *protein technology has been applied in only a few instances to cytokines and growth factors. Fully active *IL-2- and IL-10-IgG fusion proteins have been constructed, but data are not available for other cytokines *and growth factors.

* During Phase I, we will create recombinant fusion proteins comprising GH, G-CSF or EPO fused to *the Fc (Hinge- CH2-CH3) or complete heavy chain (CH1-Hinge- CH2- CH3) domains of human IgG1. *The fusion proteins will be expressed as secreted proteins from transiently transfected mammalian cells or *Baculovirus-infected insect cells. The proteins will be purified by Protein A or G affinity chromatography *and their bioactivities compared to the corresponding non-fusion proteins in in vitro cell proliferation *assays. Fusion proteins that retain full in vitro bioactivity will be candidates for further development.

* During the Phase II portion of the grant, we will develop manufacturing processes to produce sufficient *quantities of the fusion proteins for pharmacokinetic analyses and for testing in animal disease models. *Our goal is to create fusion proteins that are equal or superior to the natural proteins in stimulating *biological activities in vivo, but which require less frequent dosing, on the order of once every two to four *weeks, rather than daily or every other day. Previous studies suggest it should be possible to achieve this *goal by fusion of the proteins to the heavy chain domain of human IgG1.

* The primary goal of the Phase I portion of the grant is to identify one or more IgG fusion proteins of *GH, G-CSF or EPO that possesses wild type in vitro biological activity. The specific tasks involved are:

*1. Clone cDNAs encoding GH, G-CSF and EPO and fuse DNA encoding these proteins to DNA encoding *the Fc or complete heavy chain (C_H) region of human IgG1.

*2. Clone DNA encoding the fusion proteins into a mammalian cell or insect cell expression vector.

*3. Transiently transfect mammalian cells with DNA encoding the fusion proteins and purify the secreted *fusion proteins to homogeneity using Protein A or G affinity chromatography, followed by other *chromatographic procedures, if needed. Alternatively, express the fusion proteins in insect cells and *purify the secreted proteins as described above.

*4. Characterize the fusion proteins by polyacrylamide gel electrophoresis under reducing and non-reducing *conditions to determine whether the fusion proteins are dimeric. Determine the effective sizes of the *fusion proteins using size-exclusion chromatography.

*5. Measure bioactivities (EC_{50}s) of the fusion proteins using appropriate GH-, G-CSF-, and EPO- *responsive mammalian cell lines in culture. Bioactivities of the fusion proteins will be compared to the *bioactivities of the non-fused proteins.

* Fusion proteins that retain full biological activity will be candidates for further development during the *Phase II portion of the grant. These studies will entail:

*6. Develop stable cell lines expressing the fusion proteins and purify sufficient quantities of the fusion *proteins for animal studies.

*7 Perform pharmacokinetic experiments to demonstrate increased circulating half-lives of the fusion *proteins.

*8 Perform experiments in animal disease models to compare the relative efficacies of the fusion proteins to *the corresponding non-fusion proteins.

*B. Significance

* There is considerable interest on the part of patients and healthcare providers in the development of *low cost, long-acting, "user-friendly" protein therapeutics. Proteins are expensive to manufacture and *unlike conventional small molecule drugs, are not readily absorbed by the body. Therefore, proteins must *be administered by injection. Most proteins are cleared rapidly from the body, necessitating frequent, *often daily, injections for optimum effectiveness. This is the case for GH and G-CSF. Some proteins such *as erythropoietin (EPO) are effective when administered less often (three times per week for EPO) but this *is due to the fact that the proteins are glycosylated, which requires that they be produced using expensive *mammalian cell expression systems. Patients dislike injections, which leads to reduced compliance and *reduced drug efficacy. The length of time an injected protein remains in the body is finite and is *determined by the protein's size and whether or not the protein contains covalent modifications such as *glycosylation. Circulating concentrations of injected proteins change constantly, often by several orders *of magnitude, over a 24 hour period. Rapidly changing concentrations of protein agonists can have *dramatic downstream consequences, at times understimulating and at other times overstimulating target *cells. Similar problems plague protein antagonists. These fluctuations can lead to decreased efficacy and *increased frequency of adverse side-effects for protein therapeutics. The rapid clearance of recombinant *proteins from the body significantly increases the amount of protein required per patient and dramatically *increase the cost of treatment. The cost of human protein pharmaceuticals is expected to increase *dramatically in the years ahead as new and existing drugs are approved for more disease indications. *Within 10 years the sequence of the human genome will be known, unleashing a flood of potential new *protein therapeutics. Current word-wide sales of protein therapeutics are in excess of $10 billion annually *and are growing at a greater than 10% annual rate. Thus, there is a strong need to develop protein *delivery technologies that lower the costs of protein therapeutics to patients and healthcare providers. One *solution to this problem is the development of methods to prolong the circulating half-lives of protein *therapeutics in the body so that the proteins do not have to be injected frequently. This solution also *satisfies the needs and desires of patients for protein therapeutics that are " user-friendly", i.e., protein *therapeutics that do not require frequent injections.

***Extending protein half-life by fusion to human IgGs**

* A second method that has been used to prolong the circulating half-lives of proteins is to use *recombinant DNA technology to covalently fuse the protein of interest to a second protein that naturally *has a long circulating half-life. One protein that has a long circulating half-life and which has been used *to create numerous fusion proteins is human IgG1. IgG1 is the most common immunoglobulin in serum *(70% of total IgG), has a serum half-life of 21 days (Capon et al., 1989).

* Human IgGs have a multidomain structure, comprising two light chains disulfide-bonded to two heavy *chains. Each light chain and each heavy chain contains a variable region joined to a constant region. The *variable regions are located at the N-terminal ends of the light and heavy chains. The heavy chain *constant region is further divided into CH1, Hinge, CH2 and CH3 domains. The CH1, CH2 and CH3 *domains are discreet domains that fold into a characteristic structure. The Hinge region is a region of

*considerable flexibility. The various heavy chain domains are encoded by different exons in the IgG genes *(Ellison et al., 1982).

* Proteins have been fused to the heavy chain constant region of IgGs at the junction of the variable and *constant regions (thus containing the CH1-Hinge-CH2-CH3 domains - referred to as the C_H domain) and *at the junction of the CH1 and Hinge domains (thus containing the Hinge-CH2-CH3 domains - referred to *as the Fc domain). The IgG heavy chain fusions create larger proteins that are expected to have longer *circulating half-lives. IgG1 heavy chains normally form disulfide-linked dimers through cystine bonds *located in the Hinge region. Since the Hinge region will be present, and unaltered in both of the proposed *fusion proteins, we expect the fusion proteins will be dimeric. For some proteins such as the extracellular *domains of tumor necrosis factor receptors, dimer formation has provided unexpected benefits because the *dimeric proteins were found to possess increased affinities for their ligands (Mohler et al., 1993). GH, G-*CSF and EPO are believed to act as monomers and there is no evidence to suggest that dimerization will *improve activity. In fact, it is possible that dimerization with decrease bioactivity of the proteins due to *interference with the proteins binding their cell surface receptors. Whether this is the case is one of the *questions we expect to answer during Phase I. We believe the answer to this question will be the crucial *part of the grant that will determine ultimate success of the project for we believe there is sufficient *evidence with other proteins to suggest that it is highly likely the IgG fusion proteins will have longer *circulating half-lives than the non-fused proteins.

* The fusion proteins under development will be non-natural proteins and potentially immunogeneic in *humans. We hope to minimize this possibility by using human IgG1 as the fusion partner rather than an *IgG domain from another species. Thus the entire protein will be of human origin. The junction *sequences joining the two proteins will be the only "non-natural" sequence in the fusion proteins. Any *non-natural sequences can be removed at a later time, if warranted, by <u>in vitro</u> mutagenesis, as was done *with a CD4-IgG fusion protein (Capon et al., 1989). The one IgG fusion that has been studied in detail, a *fusion of a TNF receptor to the Fc region of human IgG1, has proven to be non-immunogenic in humans *(Moreland et al., 1996; 1997). Thus there is evidence to suggest that IgG fusion proteins will be non-*immunogenic.

Figure 3. Amino acid sequence and structural organization of human EPO. N-linked and O-linked glycosylation sites are underlined.

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-27 signal sequence      -1
MGVHECPAWLWLLLSLLSLPLGLPVLG

1        /9 A-HELIX  22/            A-B loop
APPRLICD SRVLERYLLEAKEA ENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQ

/59  B-HELIX    76/  B-C loop    /90  C-HELIX   107/ C-D loop
QAVEVWQGLALLSEAVLR GQALLVNSSQPWE PLQLHVDKAVSGLRSLTT LLRALGAQ

    C-D loop    /132   D-HELIX    152/          166
KEAISPPDAASAAPLR TITADTFRKLFRVYSNFLRGK LKLYTGEACRTGDR
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EPO Background

* EPO is the hormone primarily responsible for stimulating erythropoiesis or red blood cell formation. *EPO acts on immature red blood cell precursors to stimulate their further proliferation and differentiation *into mature red blood cells. Recombinant human EPO is used to restore red blood cell production in *patients with anemia resulting from renal failure, chemotherapy and drug complications. EPO recently *received FDA approval for stimulating red blood cell formation in patients undergoing certain types of *elective surgeries. U.S. sales of EPO exceeded $1 billion and world-wide sales exceeded $2 billion in *1996.

* Human EPO is a 35-39 kDa glycoprotein secreted by the adult kidney. The mature human protein *contains 166 amino acids and is heavily glycosylated. The sequence of human EPO is shown in Figure 3. *The primary sequence of EPO is highly conserved among species (greater than 80% identity). Sugar *groups account for greater than 40% of the protein's mass. Human EPO contains three N-linked *glycosylation sites and one O-linked glycosylation site (underlined in Figure 3). The N-linked *glycosylation sites are conserved in different species whereas the O-linked glycosylation site is not. *Proper glycosylation of the N-linked glycosylation sites in EPO extends the protein's circulating half-life. *in the body and improves the protein's performance in animal disease models (Fukada et al., 1989; Spivak *and Hogans, 1989; Delorme et al., 1992).

* The extensive glycosylation of EPO has prevented the protein's crystallization, so the X-ray structure *of the protein is not known. The amino acid sequence of EPO is consistent with the protein being a *member of the GH supergene family (Bazan, 1991; Mott and Campbell, 1995) and mutational studies *support this view of EPO's structure (Boissel et al., 1993; Wen et al., 1994). Amino acids predicted to *comprise the alpha helices A-D in EPO are shown in bold-faced type in Figure 3. Human EPO contains *four cysteine residues. The disulfide assignments are Cys7 to Cys161 and Cys29 to Cys33.

* Amino acids in EPO important for receptor binding have been identified through mutagenesis *experiments and reside primarily in presumptive helices A, C and D (Boissel et al., 1993; Wen et al., *1994; Matthews et al., 1996). An important finding relevant to this proposal is that fusion of a six amino *acid poly-histidine tag to the C-terminus of EPO does not interfere with EPO bioactivity (Boissel et al., *1993). This result suggests that other C-terminal fusions, such as those proposed in this grant *application, also will be active.

* Only a single cell surface receptor for EPO has been identified (D'Andrea et al., 1989). It is believed *that EPO dimerizes its receptor in a manner similar to the same way GH dimerizes its receptor *(Cunningham et al., 1991; de Vos et al., 1992; Matthew's et al., 1996).

***Commercial opportunity**

* Recombinant GH, G-CSF and EPO are three of the top five selling protein pharmaceuticals in the *world, with combined world-wide sales exceeding $4 billion in 1996. Recombinant human GH is used to

*treat short stature and recently received FDA approval for treating cachexia in AIDS patients.
*Recombinant human G-CSF and EPO are used to treat neutropenia and anemia, respectively. EPO
*recently received FDA approval for stimulating red blood cell formation in patients undergoing certain
*types of elective surgeries. Recombinant GH and G-CSF are currently administered by daily subcutaneous
*injection; whereas recombinant EPO is administered by thrice weekly intravenous (dialysis patients) or
*subcutaneous (non-dialysis patients) injections. Novel GH, G-CSF and EPO analogues with longer in vivo
*half-lives will allow the same amount of recombinant protein to be administered less frequently. Based
*upon pharmacokinetic studies with other IgG fusion proteins, it should be possible to administer GH-, G-
*CSF- and EPO-IgG fusion proteins once every two to four weeks and maintain effective circulating doses.
*Less frequent dosing should reduce the amount of protein used by patients by more than an order of
*magnitude, with cost savings potentially measured in the hundreds of millions of dollars per year. In vivo
*effectiveness of the proteins might be improved because circulating levels of the proteins will be more
*constant. The need for less frequent injections, coupled with improved efficacy, will increase patient
*compliance and quality of life.

***D. Experimental Procedures and Methods**

***Cloning of cDNAs encoding GH, G-CSF and EPO**

* A cDNA encoding GH will be amplified by PCR from commercially available single-stranded cDNA
*prepared from human pituitaries (available from CLONTECH, Inc.). A cDNA encoding human G-CSF
*will be purchased from R&D Systems or amplified using PCR from mRNA isolated from human
*carcinoma cell lines such as U87-MG (available from the American Type Culture Collection) known to
*express G-CSF constitutively (Nagata, 1994). A cDNA encoding EPO will be cloned using PCR from
*single-stranded cDNA prepared from human adult kidney or fetal kidney or liver (available from
*CLONTECH, Inc.). PCR primers will be designed based upon the known sequences of GH, G-CSF and
*EPO. Alternatively, synthetic cDNAs encoding full-length GH, G-CSF and EPO will be assembled from
*overlapping oligonucleotides. All clones will be verified by sequencing. The cDNAs will be designed to
*include the N-terminal signal sequences required for secretion of the proteins from the cell. The cDNAs
*also will be designed to delete the termination codon and add an in-frame unique restriction site to
*facilitate joining to DNA sequences encoding the IgG1 C_H or Fc domains. The latter changes will be
*included in the reverse primers used for PCR amplification or in oligonucleotides used to assemble the
*genes. The forward primers will include an optimized Kozak sequence (GCC(A/G)CCATGG), where the
*underlined ATG is the initiator methionine of the protein) for efficient translation of the proteins in
*mammalian cells (Kozak, 1991).

***Cloning of human IgG1 C_H and Fc domains**

* The Fc and C_H domains of human IgG1 will be cloned using PCR to amplify the appropriate sequences
*from single-stranded human leukocyte cDNA (available from CLONTECH, Inc.). PCR primers will be
*based upon the known sequence of IgG1 DNA (Ellison et al., 1982). The fusion points will be serine at
*position 1 of the CH1 domain and aspartic acid at the beginning of the Fc domain (Ellison et al., 1982).
*Convenient restriction enzyme sites will be incorporated into the PCR primers to facilitate subcloning into
*plasmids and fusion protein construction.

Creation of fusion proteins

GH-, G-CSF- and EPO- IgG fusion proteins will be assembled in plasmid pUC19, sequenced and subcloned into the mammalian cell expression vector pcDNA3.1, available from Invitrogen, Inc. pcDNA3.1 can be used for both transient transfection and stable transformation of a variety of mammalian cells. The plasmid contains a polylinker for cloning target genes downstream of the strong cytomegalovirus promoter, an SV40 origin of replication for high copy number replication in COS cells and selectible markers for growth in bacteria (ampicillin resistance) and mammalian cells (G418 resistance). Plasmid DNAs will be isolated using commercially available kits (e.g., Qiagen, Inc.) and used to transfect monkey COS cells in vitro. COS cells will be plated in 10 cm diameter tissue culture dishes and transfected the next day with appropriate plasmids using well established procedures (Bebbington, 1996; Linsley et al., 1991b). Following a 24 hour grow-out in serum-containing media, the cells will be washed extensively to remove serum (which could interfere with purification of the IgG fusions by affinity chromatography) and grown for an additional 24-72 hours in serum-free media. Conditioned media will be collected, concentrated and passed through a Protein A affinity column to purify the IgG fusion proteins. Human IgG1 (through the heavy chain constant region) binds tightly to Protein A whereas contaminating, residual bovine IgGs, which may be present due to use of bovine serum for initial cell growth, bind poorly to this resin (Pierce Immunochemical Reagents Catalogue). Bound proteins will be eluted from the column with low pH buffer, immediately neutralized with Tris base and dialyzed. If needed, the IgG fusion proteins will be purified further using other chromatographic methods such as ion-exchange, hydrophobic interaction or size-exclusion chromatography. Protein concentrations will be determined using commercially available protein assay kits (available from Bio-Rad Laboratories). Typical yields of other IgG fusion proteins isolated from transfected COS cells are in the range of 1 mg/liter of conditioned media (Linsley et al., 1991a).

If mammalian cell expression of the fusion proteins is not successful, we will express the fusion proteins in insect cells as secreted proteins. cDNAs encoding the fusion proteins will be cloned into commercially available vectors, e.g., pVL1392 from Invitrogen, Inc. and used to infect insect cells. Initially, we will attempt expression of the fusion proteins using the naturally-occurring human signal sequences since human signal sequences typically function in insect cells. If we find that one or more of the fusion proteins is not secreted efficiently, we will subclone the fusion protein into the expression plasmid pMELBAC (available from Invitrogen), which contains a signal sequence from the Honeybee mellitin protein and has been used to secrete mammalian proteins from insect cells. We will construct the appropriate expression plasmids and then contract the insect cell expression work with the University of Colorado Health Sciences Center Core Insect Cell Expression laboratory, which performs this work on a fee for service basis. Insect cells will be infected with recombinant viruses in media containing serum, grown for 24 hours, washed and grown for several days in serum-free media. Aliquots of the conditioned media will be collected on a daily basis and analyzed for the presence of the secreted proteins by SDS-PAGE, followed by Western blots using appropriate antisera. Alternatively, secretion of the fusion proteins will be detected and quantitated using ELISA assays specific for GH, G-CSF and EPO (R&D Systems, Diagnostic Systems Laboratories). Fusion proteins will be purified from conditioned media using Protein A affinity chromatography, as described above. Bound proteins will be released from the columns using low pH buffer, immediately neutralized and dialyzed. If needed, the proteins will be purified further using size exclusion and ion-exchange column chromatography procedures.

Physical characterization of IgG fusion proteins

The IgG fusion proteins will be characterized by SDS-PAGE in the presence and absence of a disulfide-reducing agent to determine their relative molecular masses and to determine whether they exist as disulfide-linked dimers. Based on studies with other IgG fusion proteins, we expect the proteins to exist as disulfide-linked dimers. Dimerization occurs through cysteine residues in the IgG1 heavy chain

*Hinge region, which is present in all the constructs. The apparent molecular masses of the proteins also
*will be determined by size-exclusion chromatography.

*In vitro evaluation of EPO-IgG fusion proteins

* EPO-IgG fusion proteins will be tested in cell proliferation assays using the EPO-responsive cell lines *UT7-epo (Wen et al., 1994) or TF1 (available from the American Type Culture Collection) to measure *specific activities. Dr. H.F. Bunn (Brigham and Women's Hospital, Boston, MA) has agreed to provide us *with the UT7-epo cell line. Cells will be plated in 96-well microtiter plates with serial 3-fold dilutions of *EPO, EPO-IgG fusion proteins, human IgG1 or buffer. Assays will be performed in triplicate. After 1-3 *days in culture, the cells will be washed, incubated for 4 h with ^{3}H-thymidine and harvested for *determination of incorporated radioactivity by scintillation counting. Alternatively, we will develop a *nonradioactive dye uptake assay employing MTT (Sigma) to measure cell proliferation/viability. For 8these assays, cells will be grown for 2-4 days in the presence of EPO, EPO-IgG fusion proteins, human *IgG1 or buffer before treatment with MTT. The cells will be solubilized, incubated overnight to allow *color formation and absorbance of the plates read the next day using a microtiter plate reader. The EC_{50} *will be determined for each protein. Assays will be performed at least three times for each protein and *with triplicate wells for each data point. EC_{50} comparisons will be used to compare the relative potencies *of EPO and the EPO-IgG fusion proteins. Fusion proteins displaying similar optimal levels of stimulation *(90% or more) and EC_{50} values comparable to EPO (within 2-fold) will be considered for further study.

*Phase II - In vivo evaluation of GH-, G-CSF- and EPO-IgG fusion protein candidates.

* We expect that the Phase I experiments described above will allow us to determine whether the GH-, G-*CSF- and EPO- IgG fusion proteins possess bioactivities comparable to that of GH, G-CSF and EPO in *in vitro assays. Demonstration that at least one of the IgG fusion proteins possesses wild type in vitro *bioactivity will be the criterion used for successful completion of Phase I. Fusion proteins that retain full *activity in in vitro assays will be candidates for further development. If both the C_H and Fc IgG fusion *proteins posess full activity, we will analyze both during Phase II to determine which performs best in *animal disease models.

*The following experiments will be performed during the Phase II portion of the grant.

Construction of stable mammalian cell lines expressing the IgG fusion proteins

* In order to obtain the large amounts of protein required for animal experiments we will need to develop *larger scale processes for expression and purification of the IgG fusion proteins using stably transformed *mammalian cells. We expect the purification scheme will be similar to the one developed for small-scale *production of the fusion proteins. For stable expression in mammalian cells, pcDNA3.1 plasmids *encoding the IgG fusion proteins will be used to stably transform DHFR$^-$ CHO or rodent NS0 myeloma *cells using G418 antibiotic resistance to select for cells expressing stably-integrated plasmid DNA. A *plasmid encoding glutamine synthetase or dihydrofolate reductase (DHFR) will be co-transfected with the *pcDNA3.1 IgG fusion protein plasmids to allow for gene amplification at a later time to increase protein *production levels. Following selection of single cells by limited dilution cloning, sublines will be *developed and screened for GH-, G-CSF- or EPO-IgG expression using GH, G-CSF, EPO and IgG *ELISA assays (R&D Systems, Diagnostic Systems Laboratories). Increases levels of protein production *can be achieved by selection using increasing concentrations of methotrexate (DHFR$^-$ CHO cells) or

*methionine sulphoximine for NS0 cells) in the growth media. Large scale mammalian cell culture work *will be contracted to the Colorado Bioprocess Center at Colorado State University, which performs this *service on a fee for service basis.

***Expression and purification of control proteins - GH, G-CSF and EPO**

* Large amounts (tens of milligrams) of non-fused GH, G-CSF and EPO will be required as controls for *the animal experiments and will have to be manufactured as well. Recombinant human GH and G-CSF *for human use are manufactured in bacteria, whereas recombinant EPO needs to be manufactured using *mammalian cells. GH will be expressed in E. coli using the STII or OmpA signal sequences to secrete the *mature protein into the periplasmic space (Hsiung et al., 1986; Chang et al., 1987). Secreted GH is *properly folded and biologically active. Following osmotic shock, the secreted protein will be purified by *conventional column chromatography methods. G-CSF will be produced as an intracellular protein in E. *coli. The recombinant protein is insoluble, but can be refolded into an active form using well known *procedures (Souza et al., 1986). Recombinant EPO will need to be manufactured using mammalian CHO *cells to ensure proper glycosylation (Delorme et al., 1982). Stable cell lines secreting EPO will be *prepared as described above for the fusion proteins. Recombinant EPO will be epitope tagged (using a *$(His)_6$ or FLAG (Kodak) tag) at its C-terminus to facilitate purification by affinity chromatography.

***Pharmacokinetic experiments with IgG fusion proteins**

* We will collaborate with researchers at the Office of Laboratory Animal Resources at the University of *Colorado Health Sciences Center to perform pharmacokinetic studies of the fusion proteins to determine *to what fusion of the proteins to the Fc or C_H domains of human IgG1 extends the in vivo half lives of the *proteins. The Office of Laboratory Animal Resources is an accredited animal research facility. These *data will guide us in designing animal experiments to determine dosing regimens to compare the *efficiencies of IgG fusion proteins to the natural proteins. Pairs of rats will receive an intravenous bolus *injection of each protein and circulating levels of the proteins will be measured over the course of 24 h. *Protein levels will be quantitated using commercially available human ELISA kits for GH, G-CSF and *EPO (R&D Systems and Diagnostoc Systems Laboratories). Additional experiments will be performed *using the subcutaneous route of administration. Similar experiments will be performed with the non-*fusion proteins. We expect to find that (1) fusion of the proteins to the Fc or C_H domains of human IgG *significantly extends the circulating half-lives of GH, G-CSF and EPO relative to the natural proteins and *(2) fusion to the larger C_H domain of IgG1 extends the circulating half-life more than fusion to the smaller *Fc domain.

***Animal disease models - general considerations**

* We will compare the relative efficacies of the C_H and Fc IgG fusion proteins in appropriate animal *disease models, as described below. Appropriate dosing schedules will be determined for each fusion *protein. We expect to find that the larger fusion protein molecule increases the circulating half-life greater *than the smaller fusion protein molecule and will require less frequent dosing. However, large proteins *may have reduced volumes of distribution in vivo; thus it is possible the larger fusion proteins may limit *bioavailability, reducing its efficacy. Animal disease models will allow us to determine if this is the case. *Once the optimum dosing schedule and fusion protein size are determined, we will compare the efficacies *of the fusion proteins to each other and to the non-fused proteins in the animal models. We expect to find *that the IgG fusion proteins produce results equal or superior to the non-fused proteins, but can be given *less frequently. We expect to find that the IgG fusion proteins are more efficacious than the non-fusion *proteins when both are administered using the less frequent dosing schedules.

***Animal anemia models to test EPO-IgG development candidates**

* In vivo bioactivities of the EPO-IgG fusion proteins will be tested using the artificial polycythemia or *starved rodent models (Cotes and Barigham., 1961; Goldwasser and Gross., 1975). In the starved rodent *model, rats are deprived of food on day one and treated with test samples on days two and three. On day *four, rats receive an injection of radioactive iron-59. Approximately 18h later, rats are anesthetized and *blood samples drawn. The percent conversion of labeled iron into red blood cells is then determined. In *the artificial polycythemia model, mice are maintained in a closed tank and exposed for several days to *hypobaric air. The animals are then brought to normal air pressure. Red blood cell formation is *suppressed for several days. On day four or six after return to normal air pressure, mice are injected with *EPO or saline. Mice receive one injection per day for one to two days. One day later the animals receive *an intravenous injection of labeled iron-59. The mice are euthanized 20 h later and the amount of labeled *iron incorporated into red blood cells determined. EPO stimulates red blood cell formation in both

*models as measured by a dose-dependent increase in labeled iron incorporated into red blood cells. In *both models we will study different dosing regimens and different times of injections to determine if EPO-*IgG is more potent and produces longer acting effects than natural EPO.

* We will contract with an accredited commercial animal testing facility or an academic laboratory to *perform these experiments. We also will identify an academic consultant with experience with these *animal models to help us in the performance of the experiments.

***Research summary:**

* Successful completion of the Phase I and Phase II studies outlined in this proposal will allow the *creation of long-acting versions of GH, G-CSF and EPO for use in treating short stature, cachexia, *neutropenia and anemia. These studies will provide valuable basic structure/function information about *three of the most important human therapeutic proteins discovered to date. In particular, these studies *should provide important data concerning the role of the C-terminus of the proteins in binding to cell *surface receptors and activating intracellular signaling pathways. More generally, knowledge gained from *these studies will expand the potential number of cytokines and growth factors for which IgG fusion *technology has been assessed. Successful development of fully active, long-lived GH, G-CSF- or EPO-*IgG fusion proteins will stimulate creation of additional cytokine/growth factor-IgG fusion proteins using *the same technology, particularly with other members of the GH supergene family. These long-lived *proteins will find applications in treating a number of chronic disease indications, including endocrine, *hematopoietic and inflammatory disorders and cancer.

EXHIBIT B

FROM

IRG: SPECIAL EMPHASIS PANEL (SEP)
BIOLOGICAL & PHYSIOLOGICAL SCIS SEP
DR. ABUBAKAR A. SHAIKH, SRA
CTR FOR SCIENTIFIC REV (PREV DRG)
6701 ROCKLEDGE DRIVE MS7892
BETHESDA MD 20892
(301) 435-1042

01/13/98

Your grant application has been received by NIH and assigned to an Initial Review Group (IRG) for scientific merit evaluation and to an Institute for funding consideration. The Initial Peer Review should be completed by 03/98 and a funding decision made shortly after the appropriate National Advisory Group meets in 05/98. For questions about the assignment, contact the Referral Office (301) 435-0715. For questions prior to the review, contact the IRG Scientific Review Administrator (SRA) named above. For questions after the review, contact the Institute listed below.

PRINCIPAL INVESTIGATOR: COX, GEORGE N   COUNCIL: 05-98
TITLE: GROWTH FACTOR CONJUGATES FOR TREATING HORMONAL DISEASES
ASSIGNMENT NUMBER: 1R43DK54561-01   DUAL: HL IRG: SEP (ZRG2 REN (1) B)

NATL INST DIABET/DIGEST/KIDNEY DIS
EXTRAMURAL ACTIVITIES, 45 6AS-37
NATIONAL INSTITUTES OF HEALTH
BETHESDA, MD 20892 (301)594-8885

COX, GEORGE N
BOLDER BIOTECHNOLOGY, INC
678 WEST WILLOW STREET
LOUISVILLE, CO 80027

FROM

PHILIP F. SMITH, PH.D.
DIR PITUITARY & NEUROENDO PROG.
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(301) 594-8816
PHONE CONTACT #: ps56z@nih.gov

04/02/98

The 1st phase of the dual review of your application (1R43DK/HL54561-01) is complete. The Initial Review Group (IRG) accorded your application a PRIORITY SCORE of 183. An IRG summary statement containing important evaluative comments and budget recommendations will automatically be sent to you in approximately 8 weeks. Until then, no specific information regarding the review will be available. However, you may call the contact number above at any time with other questions. After receiving your summary statement you may also call to discuss its contents, and for advice regarding a possible resubmission. Should a revised application be indicated, you must follow the instructions in the application kit and respond specifically to the critical comments in the summary statement.

COX, GEORGE N
BOLDER BIOTECHNOLOGY, INC
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SUMMARY STATEMENT
(Privileged Communication)

Application Number: 1 R43 DK54561-01

DUAL PROGRAM CLASS CODE: BBDQ N DUAL: HLCA
ZRG2 REN (1)

Review Group: BIOLOGICAL & PHYSIOLOGICAL SCIENCES SEP

Meeting Dates: IRG: FEB/MARCH 1998 COUNCIL: MAY 1998 4B-2
Requested Start Date: 07/01/1998

COX, GEORGE N, PHD
BOLDER BIOTECHNOLOGY, INC
678 WEST WILLOW STREET
LOUISVILLE, CO 80027

Project Title: GROWTH FACTOR CONJUGATES FOR TREATING HORMONAL DISEASES

IRG Action: Priority Score: 183
Human Subjects: 10-NO HUMAN SUBJECTS INVOLVED
Animal Subjects: 10-NO LIVE VERTEBRATE ANIMALS INVOLVED

GENDER, MINORITY, & CLINICAL TRIAL CODES NOT ASSIGNED

PROJECT YEAR	DIRECT COSTS REQUESTED	DIRECT COSTS RECOMMENDED	ESTIMATED TOTAL COST
01	86,500	86,500	100,000
TOTAL	86,500	86,500	100,000

RESUME AND SUMMARY OF DISCUSSION: This proposal is intended to prepare long acting analogs of growth hormone(GH), granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO) by conjugating with the Fc or complete heavy chain domain of human IgG-1. The methodology proposed is straight forward. Although the proposal has scientific merit, the fact that the applicant has to generate all the cDNA clones represents a significant amount of effort and it is not clear if the applicant has expertise for accomplishing the proposed work.

DESCRIPTION: Most human protein therapeutics require frequent dosing due to rapid clearance of the proteins from the body. Development of second generation protein pharmaceuticals that can be injected less frequently is of considerable interest to patients and healthcare providers. We propose to create long-acting forms of growth hormone, granulocyte colony stimulating factor and erythropoietin by fusion of these proteins to a naturally-occurring protein with a long circulating half-life. These modified proteins will possess biological activities equal or superior to the corresponding natural proteins in vivo, but will require less frequent dosing, on the order of once every two to four weeks, rather than daily or every other day. During Phase I we will construct the fusion proteins and demonstrate that they possess wild type in

vitro bioactivities. During Phase II, we will manufacture sufficient quantities of the modified proteins for testing in animal disease models. The improved characteristics of the novel proteins will reduce the amount of protein required per patient, improve patient compliance and quality of life and result in considerable cost savings to patients and healthcare providers. These proteins will find utility in treating endocrine and hematopoietic disorders, and complications of AIDS and cancer chemotherapy.

CRITIQUE 1: During Phase I, the applicant proposes to produce conjugates of growth hormone (GH), granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO) with Fc or complete heavy chain (CH) region of human IgG-1. The methodology would involve PCR amplification of GH, G-CSF and EPO sequences and ligating these sequences with cDNA corresponding to Fc and Ch domains to produce fusion proteins followed by subcloning the fusion gene in a mammalian expression vector pcDNA3.1 for transient or stable transfections in mammalian cells. Following purification, the biologic potency of the fusion protein will be tested for their ability to stimulate in vitro cell proliferation. The Phase II of the project would involve large scale isolation of the fusion protein and in vivo biologic testing.

The methodology proposed is a commonly used procedure for the construction of fusion proteins. Therefore, this aspect should proceed without great difficulty. The strategy for testing the biologic potency and serum clearance is also straight forward. The proposed experiments are technically feasible and should proceed without much problem and the investigation should be able to proceed to Phase II study.

Although the experimental strategy is straightforward, it is somewhat disconcerting that they have not acquired any of the required reagents. The applicant does not appear to have experience in cloning.

CRITIQUE 2: Recombinant proteins, such as GH, G-CSF, and Epo, are used therapeutically at present to treat short stature, cachexia, neutopenia and anemia. Current world-wide sales of such protein therapeutics is more than $10 billion, and growing at a more than 10% annual rate. In their present form, however, these agents must be administered frequently, usually by injection, due to their rapid clearance from the circulation. Among the factors that determine the clearance rate for a circulating protein are its size and types of secondary modifications, such as glycosylation. The applicant's goal, to create longer acting forms of GH, G-CSF, and Epo by expressing these as fusion proteins with the heavy chain of human IgG1, is an outstanding one. The concept has a solid scientific base: Human IgG1 has a long serum half- life, on the order of approximately 21 days and IgG fusion proteins constructed with other proteins considerably extends (e.g. [illegible] circulating half-life. Despite evident success with substantial [illegible] of several cytokines and growth factors, IgG fusion [illegible] largely remained in the laboratory realm since its incep[illegible]

An alternative approach to increase [illegible] circulating protein, with a longer history, is to c[illegible] which very effectively increases the pro[illegible] cross-linking reagents successfully used to PEGylate p[illegible] amino group of lysine residues. This becomes p[illegible] ins contain multiple

available Lys residues, resulting in heterogeneous cross-linking and a non-uniform product. Furthermore, Lys residues often play a key role in a protein's bioactive properties; consequently, PEGylation is frequently found to decrease, if not destroy, a protein's bioactivity. Indeed, the three proteins under study in this application all contain Lys residues that are known to be critical for bioactivity. Free Cys residues can also be targeted for PEGylation using a Cys-reactive PEG, but generally this involves protein engineering to replace a surface- exposed non-essential residue with a Cys. To be successful, this involves intimate knowledge of the protein's 3D structure and modeling studies so that the new Cys residue does not interfere with proper protein folding. The applicant has extensive experience with protein PEGylation, and appears fully aware of the strengths and weaknesses of the various approaches. The applicant has used 4 main criteria to develop this proposal: (1) Recombinant GH, G-CSF and Epo represent 3 of the top 5 selling protein pharmaceuticals in the world, emphasizing the commercial utility of the proposal; (2) Although their primary structures differ extensively, GH, G-CSF and Epo bear similar tertiary structures and are considered to be members of a single supergene family. Given this structural similarity, their mechanism of interaction with cognate surface receptors is also analogous; (3) IL-2 and IL-10 are additional members of the same supergene family, and IgG fusion protein technology has been applied successfully to them; and (4) It is known that improving the circulating half-lives of GH, G-CSF and Epo increases their effectiveness in vivo. These criteria are clear and form an excellent basis for the proposal.

Five specific aims are proposed for Phase I, which is budgeted for a 6 month period:

1. Clone cDNAs encoding GH, G-CSF and Epo and fuse these cDNAs to DNA encoding the Fc or CH of human IgG1. A PCR-directed cloning strategy is proposed using commercially available reagents.

All clones will be verified by sequencing. For GH, G-CSF and Epo, the cDNAs will include the N-terminal signal sequences required for secretion, and will be designed to delete the termination codon, with the addition of a unique restriction site to allow the join to the Fc or CH encoding DNA. For the latter, the fusion points will be Ser1 of the CH domain and Asp1 of the Fc domain. Efficient translation of the proteins in mammalian cells will be facilitated by the incorporation of an optimized Kozak sequence. These are all straight- forward techniques with which the PI has extensive experience, and no difficulties are anticipated. The one question that remains deals with the IgG portion of the constructs. No criteria are presented to determine how the applicant will choose the Fc or CH domain as the fusion partner for each of the constructs, or whether both forms will be constructed for each of the candidate proteins.

Clone the DNAs encoding the fusion proteins into mammalian or insect cell expression vectors.

The fusion proteins will be assembled in plasmid pUC19, sequenced and subcloned into the mammalian cell expression vector pcDNA3.1 (Invitrogen), which can be used for both transient transfection and stable transformation. This plasmid is a good choice as it contains the strong CMV promoter, an SV40 origin of

replication for high copy number and selectable markers for growth in bacteria (amp resistance) and mammalian cells (G418 resistence).

Transiently transfect monkey COS cells with the fusion protein expression vectors, purify secreted fusion protein to homogeneity using Protein A or G affinity chromatography, and other chromatographic procedures, if needed. Move to insect cell line expression, if necessary.

Transfected cells will be grown in serum-containing medium for 24 hr, and then grown for an additional 24-72 hr in serum-free medium. Media containing the secreted fusion proteins will be concentrated and applied to a Protein A column to purify the fusion proteins by binding the Fc portion of the expressed proteins. The applicant should exercise caution at this step since bovine IgGs, contained in the FBS used for the initial culture, will also bind to Protein A although not as well as the human IgGs. (The use of Protein G would be a poor choice at this step, since bovine IgGs bind avidly to this matrix.) Depending on i) the level of expression of the fusion proteins and ii) the efficiency of switching to serum-free conditions, the Protein A column may be overwhelmed with bovine IgG. The applicant will want to test this by probing the column eluate with a commercial anti-bovine Ig Ab in an EEISA or immunoblot format, since the extent of contamination may be difficult to detect on SDS gels.

If mammalian cell expression of the fusion proteins is unsuccessful, the applicant plans to move to insect cells. In this case, the appropriate cDNAs will be cloned into pVL1392 (Invitrogen). The applicant is aware of the signal sequence modifications that may be required to enable efficient insect cell expression. After constructing the expression plasmids, all the insect cell expression work will be contracted out to the Univ of Colo Health Sciences Core Insect Cell Expression Lab, with purification and analysis of the secreted proteins by the applicant, as described for the mammalian cell system.

4. Characterize the fusion proteins by SDS-PAGE under reducing and non-reducing conditions to determine whether they are dimeric. Determine the effective size of the fusion proteins using size-exclusion chromatography.

Based on studies with other IgG fusion proteins, the applicant anticipates that the fusion proteins will be expressed as dimers, linked by disulfide bonds at the Cys residues of the IgG hinge region. This property may be crucial for the following studies on bioactivity since GH, G-CSF and Epo are all thought to bind as monomers to their respective receptors, causing dimerization of the latter. It is impossible to predict whether dimerization of the proposed fusion proteins will influence their bioactivity, i.e. if the fusion proteins dimerize and poor activity is detected, steps will be taken to monomerize the fusion proteins and re- examine their

5. Measure the bioactivity (EC50) of the fusion proteins using appropriate GH-, G-CSF- and Epo-responsive cell lines in vitro, comparing the bioactivity of the fusion proteins to non-fused counterparts.

Bioactivity of the IgG fusion proteins will be assessed using published in vitro cell proliferation assays. [illegible] for each ligand will be used; for the G-CSF and Epo constructs, [illegible] are available commercially whereas, for the GH constructs, [illegible] the process of stably

transforming FDC-Plcells with human GH receptor to generate GH- responsive cells. All of the cell lines considered by the applicant respond to wild-type (i.e., non-fused) ligand in the physiological range (pg/ml).

For each ligand the details of the assay are well described and appropriately controlled, including the IgG fusion protein of interest, its non-fused counterpart, and human IgG1. Assays will be performed a minimum of 3 times for each protein using triplicate wells for each data point. The EC50 will be determined for each protein, and used to compare the relative potencies of the IgG fusion proteins. Those IgG fusion proteins that display similar optimal levels of stimulation ($90%) and EC50 values comparable to the wild-type ligand will be considered for Phase II study.

In Phase II, fusion proteins that retain full bioactivity will be developed further by creation of stable cell lines for fusion protein expression and purification of sufficient quantities of the expressed fusion proteins for animal studies. The latter will involve pharmacokinetic studies to evaluate the circulating half-lives of the fusion proteins and experiments in animal disease models--which have already been documented for each of the ligands described--to compare the relative effectiveness of the fusion proteins to their non-fused counterparts.

An invaluable strength of this proposal lies, not in the novelty of any individual aspect, but in the proposal in aggregate. None of the techniques described is unique to this proposal, but this should not be construed as a handicap. Indeed, the applicant has used available technology to formulate a solid plan with which he is well versed theoretically and practically. The innovative portion of the project is putting it all together to generate therapeutically beneficial products for which there is a large market. If successful, the results of this project should spawn the development of many other IgG fusion proteins that are similarly rewarding.

The PI is the only individual named in the proposal, with a Scientist and a Research Assistant to be determined. Dr Cox worked on C. elegans at UC Santa Cruz and received a PhD in Biology in 1980. This was followed by a post-doc in David Hirsh's lab at Univ of Colo, Boulder. Since 1983, Dr Cox has been involved in the biotech industry, principally in Discovery Research and Preclinical Development. He is well trained in molecular biology, cell culture and protein chemistry, and has had extensive experience in all facets of the proposed work.

Boulder Biotechnology is housed within the Dept of Molecular, Cellular and Developmental Biology at the Univ of Colo, Boulder, which provides a stimulating atmosphere. The facilities and equipment appear adequate to complete Phase I.

BUDGET: The budget requested is appropriate for the proposed work.

DEPARTMENT OF
HEALTH & HUMAN SERVICES
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Dr. George N. Cox
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

80027-1030 13

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
National Institutes of Health

National Institute of Diabetes and
Digestive and Kidney Diseases
Bethesda, Maryland 20892

July 24, 1998

Our Reference: 1 R43 DK54561-01

George N. Cox, Ph.D.
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

Dear Dr. Cox:

The National Diabetes and Digestive and Kidney Diseases Advisory Council, at its May 27-28, 1998 meeting, completed second level review and recommended approval of the Phase I Small Business Innovation Research (SBIR) grant application referenced above.

We are pleased to inform you that we intend to fund this application subject to resolution of the issues discussed below. As part of our administrative review, we have identified the following issues that must be addressed before an award can be made.

- o Verification of Principal Investigator's Primary Employment
- o Updated Other Support Information
- o Confirmation of Direct and Indirect Cost Award Amounts
- o Fee/Profit

Please provide updated Other Support information for yourself and all other key personnel. This information should be shown in three groups: (1) all currently active research support; (2) all applications or proposals pending review or funding; and (3) applications and proposals planned or being prepared. Include all federal and non-federal grant and contract support and specifically identify SBIR projects. For each item, give the source of funding, identifying number, project title, name of principal investigator, hours per week on the project, annual direct costs, dates of the entire period of support and a brief description of the project. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, delineate and justify the nature and extent of the scientific and budgetary overlaps or boundaries.

SBIR guidelines indicate that the primary employment of the Principal Investigator must be with the small business at the time of award and during the conduct of the proposed SBIR project. Primary employment means that more than one-half of your time must be spent in the employment of the small business. We hereby request certification that you meet this requirement. This certification may be a letter signed by both you and an authorized official of the small business. If you are also an employee of another company or institution, we require a signed statement from an authorized official of that organization, indicating that

you are or will become a less than half-time employee of that organization during the proposed SBIR project.

Effective with SBIR grant awards with budget period start dates of July 1, 1993, and beyond, grantee organizations may request a reasonable fee or profit as part of the total amount of the SBIR award. A fee or profit is part of the grant award which is $100,000 for Phase I grants. In your response letter, please include a statement declining or waiving this fee/profit, or a revised budget proposing a fee/profit. Either response must be properly countersigned by your business official.

Current SBIR Guidelines state that the total cost (direct plus indirect and fee/profit) of a Phase I award may not exceed $100,000. Our preliminary review indicates that the estimated total cost is $100,000. This is based on recommended direct costs of $86,500 plus $9,500 indirect costs and fee/profit $4,000.

Once the above issues have been addressed satisfactorily, we will proceed with issuing a Notice of Grant Award for this project. However, please be aware that this letter does not take the place of the official award notice. Therefore, any expenditures or commitments made prior to receipt of a Notice of Grant Award, are at your own risk.

The signatures below identify the Institute staff administering this award. The program director should be contacted with respect to scientific and technical aspects of the award and the grants management specialist should be contacted regarding business administration of the award and matters pertaining to PHS policies.
Additionally, the following internet websites contain information which you may find useful:

1) http://www.nih.gov/grants/funding/sbir.htm
SBIR/STTR Home Page

2) http://www.nih.gov/grants/funding/welcomewagon.htm
NIH "Welcome Wagon" Letter

Information provided in this memorandum is for officials of organizations planning to submit a grant application or receiving an award for the first time from the National Institutes of Health (NIH). The intent of this memorandum is to highlight key requirements, provide referrals to important sources of information available from NIH, and identify NIH, Public Health Service (PHS) and Department of Health and Human Services (HHS) offices having responsibility for certain administrative functions. Information available through these resources will be important to those having responsibility for the administrative and fiscal management of NIH grant and cooperative agreement awards.

3) http://www.nih.gov/grants/funding/funding.htm
Information about ongoing grant programs and special initiatives will be posted at this section. This includes application kits, guidelines for applications for various types of grants [e.g., fellowships (F32), regular research projects (R01)], and identification of appropriate contacts at the institutes and centers that make awards.

If you have any questions or if we may be of assistance, please contact us.

Sincerely,

Philip F. Smith, Ph.D.
Director, Pituitary &
Neuroendocrinology Res. Program
Div. of Diabetes, Endocrinology,
and Metabolic Diseases
45 Center Drive, MSC 6600
Bethesda, MD 20892-6600
(301) 594-8816

Ephraim Johnson
Grants Management Specialist
Grants Management Branch
Div. of Extramural Activities
45 Center Drive, MSC 6600
Bethesda, MD 20892-6600
(301) 594-8868
Fax # (301) 480-3504

EXHIBIT C

J. Lee Lab Notebook # 1

Project No.

LE Epo oligos / Book No. 97

m Page No.

BB41 = mmR

BB42 =

No Kozak

BB41 (F) - 31-mer

Bam

5' CCCGGATCCATGGGGGTGCACGAATGTCCTG 3'

NCo

13x4 = 52

9x2 = 18

70

T_m = 70

BB42 (R) 31-mer

R1

5' CCCGAATTCTATGCCCAGGTGGACACACCTG 3'

D R stop

T_m = 70°

GACAGATGACCAGGTGTGTCCACCTGGGCATA

ACTGGTCCACACAGGTGGACCCGTAT

order human fetal liver QuickClone cDNA ~~$}$~~ 6/3/98

CLONTECH

To Page No.

Witnessed & Understood by me,

Date 6/30/98

Invented by Jack

Recorded by

Date 6/3/98

6/2/98

From Page No.__

PRODUCT: Human Fetal Liver QUICK-Clone™ cDNA

CATALOG #: 7171-1

LOT#: 7100701

STORAGE BUFFER:
0.5X TE buffer

STORAGE CONDITIONS:
- –70°C
- Working portions may be stored at –20°C for up to 2 weeks in a constant temperature (not "frost-free") freezer.
- Avoid multiple freeze/thaw cycles.

SHELF LIFE:
1 year from date of receipt under proper storage conditions.

SHIPPING CONDITIONS:
Dry Ice (–70°C)

DESCRIPTION:
High-purity, double-stranded cDNA for rapid cloning, sequencing, or probe generation. cDNA was synthesized using an oligo(dT) primer and purified to remove interfering RNA. The cDNA has been size-selected to remove fragments smaller than ~400 bp.

CONCENTRATION: 1 ng/µl

POLY A⁺ RNA SOURCE:
normal, whole livers pooled from 2 female Caucasian spontaneously aborted fetuses, ages 22 and 26 weeks.

No further RNA source information is available.

PACKAGE CONTENTS:
- 2 vials of cDNA, each containing approximately 10 ng. Each vial is sufficient for 10 or more PCR reactions
- Applications discussion and reference list
- Complete protocol (PT1150-1)

FOR RESEARCH USE ONLY

QUALITY CONTROL DATA

The cDNA is tested for successful amplification of a 1.1-kb human β-actin cDNA fragment in 35 cycles or less using 0.5 ng of cDNA.

APPROVED BY: _____

(PA71303)

NTECH

CLONTECH Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA
TEL: 800/662-CLON • 415/424-8222 • FAX: 800/424-1350 • 415/424-1064 • e-mail: tech@clontech.com

To Page No.__

Witnessed & Understood by me,	Date	Invented by	Date
	6/30/98	Recorded by	6/5/98

Epo PCR oligos

Project No._____

Book No._____

A Division of Perkin-Elmer Cor

Customer Name	COX	
Customer Address	SANGAMO	
Phone | Fax #		
PO Reference	**Acct**	
Order Date	6/3/98	
Comments		
Protocol Options	SynPure 4.14.Mod	Purify Oligo
Sequence Name	BB41	
Sequence 5' Length:031	CCC GGA TCC ATG GGG GTG CAC GAA TGT CCT G 3'	

A:005 G:011 C:009 T:006 5:000 6:000 7:000 8:000

M:(AC) W:(AT) Y:(CT) V:(ACG) D:(AGT) N:(AGTC)
R:(AG) S:(CG) K:(GT) H:(ACT) B:(CGT) T:T or U

Clear

A Division of Perkin-Elmer Cor

Customer Name	COX	
Customer Address	SANGAMO	
Phone | Fax #		
PO Reference	**Acct**	
Order Date	6/3/98	
Comments		
Protocol Options	SynPure 4.14.Mod	Purify Oligo
Sequence Name	BB42	
Sequence 5' Length:031	CCC GAA TTC TAT GCC CAG GTG GAC ACA CCT G 3'	

A:007 G:007 C:011 T:006 5:000 6:000 7:000 8:000

M:(AC) W:(AT) Y:(CT) V:(ACG) D:(AGT) N:(AGTC)
R:(AG) S:(CG) K:(GT) H:(ACT) B:(CGT) T:T or U

Clear

To Page No._____

cssed & Understood by me,	Date 6/30/98	Invented by	Date
		Recorded by	6/5/98

Project No._____
Book No._____ TITLE Epo PCR

From Page No.___

Csp oligos in 200 λ H_2O, OD 10 λ → 1 ml

stock OD		OD_{260}	length	ε	For 10 pmole/μl
1.3	BB 41	.0070	31	20.5 pmole/μl	50 λ + 50 λ H_2O
2.7	BB 42	.122	31	36 pmole/μl	28 λ + 72 λ H_2O

PCR

H_2O	73	
10X Buffer	10 λ	
10X dNTPs	10 λ	
5' prim	2 λ	BB 41 20 pmole
3' prim	2 λ	BB 42 20 pmole
cDNA	2.5 λ	Quick-Clone Fetal liver Human
Taq	1/2 λ	↑↓ 20 x
Pfu	1/2 λ	
	TV = 100 λ	

100
27
73

Program 42

oligo T_m = 70
Use annealing of 63°

96° 3 min;
(95° 1 min; 63° 30 sec; 72° 1 min) 35
10' 72°
4° Cold

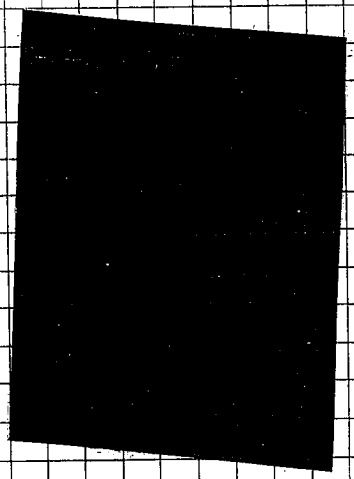
Expect ~ 600 bp

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	6/30/98	[signature]	6/5/98
		Recorded by	

TLE Epo ICR

om Page No.



Forgot to add marker

See nothing except oligo

← oligo

To Page No.

Witnessed & Understood by me,	Date 6/20/96	Invented by	Date 6/5/98
		Recorded by	

06

Project No.

Book No. TITLE EPO PCR repeat

From Page No.

GH (pCB6 diluted 50x)

	1	2	3
H_2O	73	67	71
10x PCR	10	10	10
10x dNTP	10	10	10
5' prim	20 BB41	50 BB41	3 20 BB1
3' prim	20 BB42	50 BB42	3 20 BB1
cDNA	2λ	2λ	2
Taq	1/2	1/2	1/2
Pfu	1/2	1/2	1/2

100
29
71

Try with 20λ & 50λ of prim
20 pmol 50 pmol

100
33
67

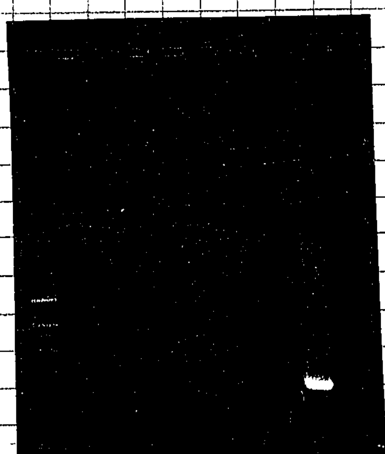
Do GH as control for Sutlers

Program 42

run 2λ of 41/42 prim on gel

T_0 = 12:11

ΦX 41 42 1 2 3



Nada again

- few oligos off so can't judge
- Reagents good cause GH worked
- Try other source

4/

To Page No.

Witnessed & Understood by me, Date 6/30/98

Invented by Joe C Date 2/9/98

Recorded by

om Page No.

- grow Hep 3B cells to isolate RNA to RT-PCR EPO
- HEP 3B's express EPO upon treatment with hypoxia, Cobalt or DFX

- grow flasks Hep3B's + 100 mM dishes

- divide in 2
  - 1/2 treat with 100 mM Cobalt chloride
  - 1/2 treat with DFX

T_0 =

- Harvest cells

didn't do this see next page

- isolate RNA with RNeasy midi kit from Qiagen
- add 4 ml RLT buffer ( )
  - vortex 10 sec
  - pass lysate thru 18-20g needle 5-10x
- add 1 vol ( ) of 70% ethanol & mix by shaking
- apply to RNeasy midi spin column in 15 ml centrifuge tube
  - close tube
  - ↓ 5 min at 3000-5000 g
  - discard flow-thru

next page

Witnessed & Understood by me,	Date 6/30/98	Invented by Joe C	Date 6/18/98
		Recorded by	

Project No._____ Book No._____ TITLE HepG2B mRNA isolation

From Page No.___

- add 3.5 ml RW1 buffer, close tube & ↓ 5 min cycle
  - discard flow-thru
- add 2.5 ml buffer RPE, spin 2 min at 3000-5000 g
  * add 4 vol of ethanol before use
- add another 2.5 ml RPE buffer, repeat 5 min
- ▲ midi column to new collection tube
  - add 150 - 250 ml RNase-free H_2O
  - let stand 5 min
  - ↓ 3 min 3-5K g's
- repeat elution step

TV =

did it - do

To Page No.___

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	6/3/98	[signature]	6/18/98
		Recorded by	

TLE Use Mini-kit Hep 3B mRNA isolation

om Page No.

- our centrifuge does not go to 3-5K g
- sucks
- use mini kit from Qiagen
  - mix up buffer 3 ml RLT
    30 λ BME
  - enough for 4×10^7 cells
    typ cells at 600 λ RLT/BME / 10^7 cells
- Trypsinize cells & PT

ETOH we have is 95% McCormick

to 70%

$(95\%)(x) = 70\%$

$x = \frac{70}{95} = 7.37$ ml → 1.0 ml H_2O

7.37 = 3.7 ml → 5 ml ~~Total~~
1.3 ml RNase H_2O
5 ml

- add 44 ml ETOH to RPT buffer
-

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
	6/30/98	Recorded by	6/18/98

Project No.

110 Book No._____ TITLE Hep3B mRNA isolat

From Page No.__

Cells from flasks
- wash HBSS
- 3 ml Trypsin / EDTA / flask
- 7 ml media + serum

TV = 10 ml × 4 flasks

CT = 170 cells in 3 fields = 57 field × 10^4 = 5.4×10^5 / ml × 10 ml = 5.4×10^6 total

- lyse in 350 λ RLT / BME per flask
- vortex 10 × each
- add another 100 λ of RLT / BME Cake extra cells

TV = 450 λ

disks 4 of each type
- use only 3 of each type
- 1.5 ml trypsin / EDTA.
  3 ml media
  TV = 14 ml
- CT 181 in 8 fields ~ 23 / field × 10^4 = 2.3×10^5 / ml × 14 ml

× 2 tubes 3.2×10^6 / tube

- lyse in 350 ml RLT / BME
- vortex 10 × each

TV = ~~450~~ 350 λ

To Page No.__

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	4/30/98	[signature]	6/18/98
		Recorded by	

Project No.

TITLE Hep 3B mRNA isolate   Book No.

From Page No.

- homogenize
  - use Qiashredder tubes
    - 700λ tube
      - flasks - consider same Type

TV = 900λ
700λ — no → 625
200λ left over 275 → to dish tubes

do so all the same

- dishes TV = 350λ
+ 200λ +75 = 275
550λ + 75 = 625

- spin 2 min per protocol in microfuge

4 tubes total

- volume each = 625λ

- add 1 volume 70% ethanol to each
= 625λ

- saw DNA floculent precipitate

TV/tube = 625
625
1350 ml

- divide to 2 RNeasy tubes
= 625λ/tube

= 8 RNEasy tube

- spin 15 sec

To Page No.

Witnessed & Understood by me, [signature] Date 6/30/98

Invented by [signature] Date 6/18/98

Recorded by

Project No.
Book No.
TITLE mRNA isolat

From Page No.

- elute each 40λ H_2O x 2 cause expect yield to be low
- TV = 80λ / tube

- Combine all the Cobalts
  TV = 320
  OD 40λ

Combine all the DFX's
  TV = 320λ
  OD 40λ

260/280 ratio = 1.9/1

40λ → 1 ml

do OD's 260/280

	260	280	C3
"C"	1.105	0.577	1.05 mg/ml = 320 ug
"D"	0.843	0.446	0.843 mg/ml = 270 ug

260/280 = 1.89/1

Total "C" cells = 5.6×10^6 x 2 = 1.12×10^7 flasks
+ 0.32×10^7 dish
1.42×10^7 cells

D - assume the same

∴ = 225 ug / 10^7 cells for "C"
= 190 ug / 10^7 cells for "D" = 84% of "C"

To Page

Witnessed & Understood by me, [signature] Date 6/30/98
Invented by [signature] Recorded by Date 6/18/98

Project No.______

RT-PCR EPO — Book No.______

Page No.___

- Dan D. [illegible] do RT-PCR using both "C" and "D" ~~oligos~~ RNAs
  - use oligos to prime
  - 2nd set w/ random hexamers to prime

- used robocycler to try different anneal conditions

- Results: Nada

Dan noticed oligos had precipitate & looked funny
- rediluted oligos before doing above RT-PCR

- reordered oligos 6/22/98 called BB45 & BB46
  60 056

- Dan did RT-PCR using GH-Receptor oligos since Hep3B is liver cells
  Result was good yield of right size band
  ∴ RNA is good

either EPO not present or bad oligos.

6/24/98

Noticed reverse oligo can form 6 bp stem at 3' end could be messing up PCR

- order new reverse oligo BB47 that deletes problem area
- could try hot start PCR ~~to~~ w/ oligo I have

To Page No.______

Witnessed & Understood by me,	Date	Invented by / Recorded by	Date
[signature]	6/30/98	[signature]	6/22/98

Dan Doherty Notebook #4

Project No.
Book No.

RT-PCR TO CLONE EPO

JUNE 19, 1998

RT-PCR OF TOTAL RNA FROM HG LIVER CELL LINE from (JC) LABELED "C" & "D"

TRY TO PCR EPO

1ST STRAND RXNS: AS PER BMB KIT SEE WB #2 p 146

RXN "C"
10ul BMB RxnBuffer 1
20ul 25mm $MgCl_2$
10ul dNTP Mix
5ul RNAsin
36ul H_2O
4ul AMV RT
5ul RNA "C" (5.25ug)

RXN "D" DHD
10ul BMB Rxn Buffer
20ul 25mm $MgCl_2$
10ul dNTP Mix
5ul RNAsin
35ul H_2O
4ul AMV RT
6ul RNA "D" (5.06ug)

SPLIT INTO 2 x 45ul ALIQUOTS

ADD to each 45ul, 5ul (1.6ug) Random Hexamers or 5ul BB42 (50pmol) → EPO specific primer

Rxns = C-HX, D-HX, C-42, D-42

Incubate @ room temp 10min
@ 42°C, 60min
@ 99°C - 5min
@ 4°C - 5min go to PCRs

To Page No. 16

Witnessed & Understood by me, Date 7/24/98

Invented by: Date 8/26/98

Recorded by

From Page No. 15

JUNE 19, 1998

PCRs -

30ul C-HX 1st STRD
43ul H_2O
0.8ul BB42 (~30pmol)
1.5ul BB41 (~30pmol)

30ul D-HX 1st STRND
43ul H_2O
0.8ul BB42 (~30pmol)
1.5ul BB41 (~30pmol)

30ul C-42
1.5ul BB41 (~30pmol)
43.5ul H_2O

30ul D-42
1.5ul BB41
43.5ul H_2O

TO EACH TEMPLATE/OLIGO MIX ADD 75ul PCR MIX:
- 56ul 10x BMB Buffer
- 14ul 25mM $MgCl_2$
- 14ul Gelatin
- 262ul H_2O
- 3.5ul Taq Pol (17.5u)
- 1.5ul Pfu Pol (3.75u)

PCR - STRAT ROBOCYCLER

96°C 3MIN

95°C 1 MIN
(53, 58, 63)°C 75 sec
72°C 75 sec
} DHP 25 35 cycles

HOLD 6°C

Distributed 150ul of each RXN into 3 x 50ul Aliquots
Anneal @ 53°, 58°, or 63°C

To Page No. 18

Witnessed & Understood by me,	Date	Invented by	Date
Joe C	7/21/98	[signature] / Recorded by	6/26/98

Project No.

LE Book No. 17

m Page No. 16

JUNE 19, 1998

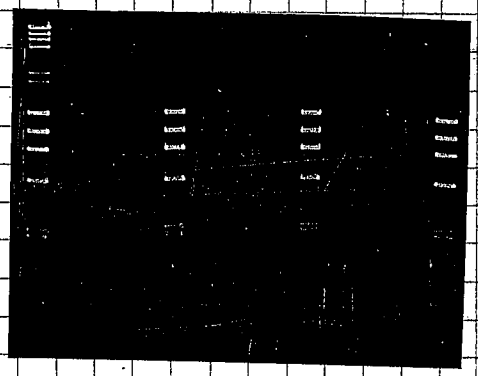
PULL PCRs + RUN OUT - look for ~600 bp band = EPO product

1.5% Az SK 1X-TBE 125V

START 2:50
STOP 4:30

LANE

1	λ/H + ΦX
2	C-Hx (53°C) 5/50ul
3	D-Hx "
4	C-42 "
5	D-42 "
6	ΦX
7	C-Hx (58°C) 5/50ul
8	D-Hx "
9	C-42 "
10	D-42 "
11	ΦX
12	C-Hx (63°C) 5/50ul
13	D-Hx "
14	C-42 "
15	D-42 "
16	ΦX



NO PRODUCT ANY SIZE ANY RXN - OLIGOS?, also failed to PCR EPO from human fetal liver (J. C. rejuvet)

RNAs? poly A intact?

1ST STRAND RXN?

6/20

TRY to PCR gh receptor from C-Hx + D-Hx to control for A+ RNA + 1ST STRAND RXN

To Page No. 18

nessed & Understood by me, Joe Cf	Date 7/22/98	Invented by [signature] Recorded by	Date 6/26/98

Project No.______
Book No.______ TITLE ______

From Page No. 17

JUNE 20, 1998

① PCRs —

3.0ul HX1 (1st strm of rabbit At NB1 p —) rab GH-R positive
1.5ul BB3 (10 pmol)
1.5ul BB4 (10 pmol)
12ul H_2O

BB4 = hu GH-R 3' rev pr
BB36 = rab " " 3' rev pr
BB3 = 5' primer (hu = rab seq)

② 10ul C-Hx
1.5ul BB3
1.5ul BB4
12ul H_2O

③ 10ul D-Hx
1.5ul BB3
1.5ul BB4
12ul H_2O

④ 10ul Hx1
1.5ul BB3
1ul BB36 (10p
12.5ul H_2O

TO MIXES 1, 2, 3, 4 ADD 25ul PCR MIX:

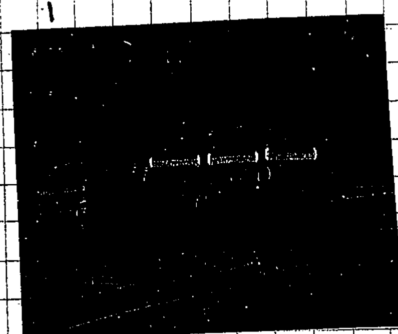
16ul 10x BMB Buffer, 4ul $MgCl_2$, 2ul gelatin, 76.5ul H_2O, 1ul Taq Pol, 0.5ul Pfu Pol

PCR — prgm 27 Amplification see NB1 p

RUN OUT PRODUCTS:

1% Ag SK, 1X TBE
150V,

START - 3:50 STOP - 4:20



1 λ / H + ΦX
2 PCR - 1 10ul/50ul
3 " 2
4 " 3
5 " 4
6

C-Hx, D-Hx BOTH GIVE EXPECTED RECEPTOR BAND. 1. 50— At OK + 1st strand OK, OLIGO PROBLEM

TRY NEW OLIGOS 7/1

To Page No.

Witnessed & Understood by me, [signature] Date 7/2/98

Invented by [signature]
Recorded by

Date 6/26/98

34

Project No.______
Book No.______ TITLE ______

From Page No. 18

JULY 1, 1998

TRY AGAIN TO PCR EPO FROM Fetal liver cDNA and HEP3B cell line ssRNA (JC) (Clontech)

NEW OLIGOS - BB45 = NEW SYNTHESIS OF 5' EPO primer FROM JC

BB47 = NEW SEQ DESIGN 3' EPO PRIMER

PCRs

(1) 10ul HX Primed Hep3B RT 1st STRAND (pooled 5ul "C" + 5ul "D")
4ul 10x BMB Rxn Buff
1 ul gelatin
1 ul BB45 (10pmol)
1 ul BB47 (10pmol)
32 ul H_2O
0.5ul Taq Pol (Pro) 2.5u
0.5ul Pfu (Strat) 1.25u

(ANNEAL @ 63°C)

PCR
Robocycler
95°C 3MIN
35x [95°C 1MIN; (59, 63, or 67°C) 75 sec; 72°C 1MIN]
72°C 10MIN
Hold 6°C

(2) 15ul 10x PCR Buffer (Pro)
9 ul 25mm $MgCl_2$
15ul 10x dNTPs
3ul BB45 (30 pmol)
3ul BB47 (")
100.5ul H_2O
(3ng) 2ul hu fetal liver cDNA Clontech
1ul Taq Pol (Pro) 5u
0.5ul Pfu (Strat) 1.25u

START 1:45
↳ 4:00

↳ SPLIT INTO 3 RXNs 50ul each:

for annealing steps @ 59, 63, 67

To Page No. 3

Witnessed & Understood by me, [signature] Date 8/6/98

Invented by [signature] Date 7/21/98

Recorded by

Project No.________

TITLE____ Book No.________

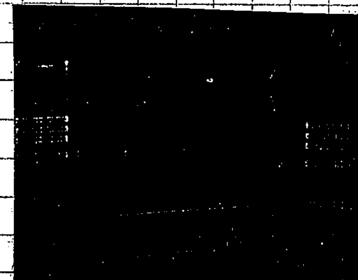
From Page No. 34

JULY 11, 1998

RUN OUT PCRs — 1.2% Ag SK 1x TBE

LANE		
1	λ/H + ΦX	
2	PCR-1 C/D H/x primed 5/50ul	
3	PCR-2 (59) Fetal liver cDNA 5/50ul	
4	" " (43) (")	
5	" " (67) (")	
6	ΦX	

120V START - 4:05 STOP - 4:35



PCR-1 gives ~ 600bp band - which is approx size expected

all others give no product -

GO AHEAD WITH CLONING FROM HEP3B product

- CLEAN UP PCR-1 with Qiagen PCR cleanup
  Elute 50ul EB

Digests:

50ul PCR1 cleanup	2ul pUC19 (2ug)
10ul 10x RE buffer (NEB)	10ul 10x NEB RE Buffer
36ul H_2O	84ul H_2O
2ul BamHI 40u	2ul BamHI
2ul EcoRI 40u	2ul RI

@ 37°C ~ 2:45 → 3:45p add 2ul CIP (Pro) 2u to pUC19 Digest

~ 4:45 PULL clean up w Q PCR cleanup, elute 40ul EB
STORE -20°C

To Page No. 36

Witnessed & Understood by me, [signature] Date 8/6/98

Invented by [signature] Date 7/21/98

Recorded by

Project No.______

Book No.______ TITLE ______

From Page No. 35

JULY 2, 1998

SET UP LIGATIONS:

Lig ① D200 7/2 @15°C DHD

8 ul pUC19 BamHI + RI + CIP ~5:30p 7/2
DHD 4 ul 5X lig Buffer (BRL)
5 ul H_2O
2 ul PCR frag (43247) x BamHI
1 ul T4 ligase (BRL) 1 Weiss u

Lig ② 7/2

8 ul pUC19 BamHI + RI + CIP
4 ul 5X lig Buffer
7 ul H_2O
1 ul T4 ligase

7/3 PUR ligs Transform w 10 ul, store 10 ul -20°C

Tfm - pUC19 1 ng/10 ul, lig ① + lig ② 10 ul + BLANK 10 ul EB
VS 100 ul COMP XL1 Blue p. 37

T=0 ADD 100 ul comp cells to DNA on ice
=10 Heat Block 42°C, 45 sec,

CHILL ON ICE, dil 1/10 w SOC, inc 37°C ~60 min

PLATE undiluted 100λ, 10λ, 1λ : pUC19 LB/Ap100
1/2 " 100λ ; lig ①, lig ②, BL

CFG lig ①, ② + BLANK, Resus 100 ul + PLATE out LB...
@37°C ON ~3p 7/2

To Page No. 3..

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	8/6/98	[signature] Doherty Recorded by	7/21/98

11

TITLE COMP CELLS : XL1 Blue Project No.____ Book No.____

From Page No.__

JULY 3, 1998

FRESH LB ON XL1 Blue → freeze 1ml as 130B13°

dil 1/10 A_{600} = .445

inoc 50ml x2 in 250ml SHAKES - @37°C

dil 1/500 0.2ml in 100ml SOC, SPLIT 2X

	A_{600}	
T=0 ~9:00a	~.01 (ASSUMED)	
10:40a	① - .085	② - .091
11:10	- .220	- .233
11:30	- .381	- .407

↳ PULL CULTS, POOL, CHILL ON ICE (50ml x2) FALCON TUBE ~15 MIN -

* CFG ~ 5500 RPM, 10 MIN IEC MP4R (Rotor 854) ~3500xg
* RESUS in 20ml ICE COLD TFB I (NB1 p82)
* HOLD ON ICE 15 MIN
* CFG AS ABOVE (6min instead of 10)
* RESUS in 2ml x2 I COLD TFB II - POOL BOTH PELLETS
* HOLD ON ICE ~15 MIN
* DISTRIBUTE 2x 0.2ml ALIQUOTS in EPPS ON ICE
* freeze in POWDERED DRY ICE
* STORE @ -80°C

TEST COMP VS pUC19 (p 37)

pUC19 → 1.35x10^{7} trans/ug OK NOT SPECTACULAR

To Page No.____

Witnessed & Understood by me, [signature] Date 8/6/98 Invented by [signature] Recorded by Date 7/21/98

Project No.

Book No. TITLE

From Page No. 36

JULY 4, 1998

PULL TRANSFORMATIONS -

XL1 Blue Blank - 100ul uncon [illegible] ϕ cfu, clean
- " conc ϕ " , "

XL1 Blue pUC19 - 1λ - 4 cfu
10λ - 135 cfu → (10 pg) → 1.35×10⁷
100λ - TNTC

XL1 Blue Lig ① 7/2 100λ unconc → ~10³ cols + backgrnd
100λ conc → confl. large -

Lig ② 7/2 100λ unconc → ~100-200 + backgrnd
100λ conc → confl. large

PLATES WENT A BIT TOO LONG + TOO CROWDED
BACKGROUND HAZE MAKES IT IMPOSSIBLE TO
ACCURATELY SCORE -

PICK 110 ISOLATED COLS FROM LIG ① +
T.P. ONTO LB Ap100 plate -

7/5 PULL PLATE, store 4°C,
INOC 3ml LB Ap100 ON's #1-?10

7/6 ON's up OK, QSP 1.5ml, elute 50ul EB
cut + run to check for
inserts - p41

To Page No.

Witnessed & Understood by me, [signature] Date 8/6/98

Invented by [signature] Date 7/21/98

Recorded by

Project No.
Book No.

From Page No. 38

JULY 6, 1998

Digestions vs (BamHI + RI) @ 37°C

2.5ul QSP3 (1→10) p 38 — 10:15 → 11:15
5 ul 10x RI Buffer
41.5ul H_2O
0.5ul Bam 12u
0.5ul EcoRI 10u

EtOH PPT, WASH pellets, AIR DRY + RESUS in 10ul H_2O

RUN OVER Digests + NAT. plas

1% Ag, SK, 1X TBE 120V START - 12:40 STOP - 1:40

TOP		BOT	
1	λ/H 10x	1	λ 1H
2	QSP 1 x(Bam+RI)	2	QSP 1 NAT 1ul
3	2	3	2
4	3	4	3
5	4	5	4
6	5	6	5
7	6	7	6
8	7	8	7
9	8	9	8
10	9	10	9
11	10	11	10
12	pUC19 Bam+RI+CIP (4ul) + EPO PCR B+R (1ul)	12	λ 1H
(13)	LigD 7/2 (10ul)		
(14)	LigD 7/2 (10ul)		
15	λ 1H + 20x		

GEL PHOTOS p 42

To Page No. 42

Witnessed & Understood by me, [signature] Date 8/6/98

Invented by [signature] Date 7/21/98

Recorded by

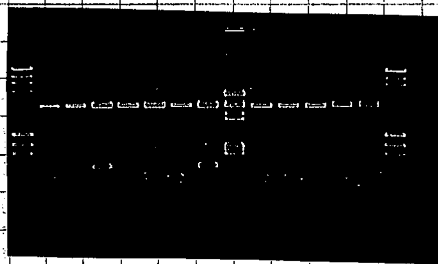
Project No.______
Book No.______ TITLE ______________________

From Page No. 41

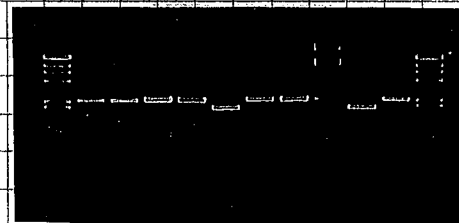
JULY 6, 1998

TOP BOTTOM

1



1



WORKS OK 7/10 have insert Bam-RI ~ 600 bp
2/10 no insert
1/10 multiplasmid?

#2,4 have also slightly ≥ 600 bp material?

						conc
OD —	#3	4 → 400 ul	A_{260} =	.084	.32 ug/ul	
	#7	" "	" =	.068	.32	"
	#10	" "	" =	.032	.16	"

DNA out for single #3, 7, 10

SHIP 3, 7, 10 to MMR for SEQS vs M13rev & M13(-20)

7/15 #10 SEQ CONFIRMED

GROW UP TO FREEZEAWAY — pBBT131 in XL1Blue

pBBT131 = pUC19::epoE10

To Page No.___

Witnessed & Understood by me, [signature] Date 8/6/98

Invented by [signature]
Recorded by
Date 7/21/98

EXHIBIT D

Monday March 3, 1997

To: Dr. Frank Bunn
Hematology / oncology Division
Brigham + Womens Hospital
Harvard Medical School

FAX: 617-739-0748
Tel: 617-732-5841

From: Joe Cox
Bolder Biotechnology
Tel: 303-665-6530

Dear Dr. Bunn,

Monday and Wednesday are very busy days for me so I will call Tuesday morning. I will try for between 9 and 9:15 AM your time. If I miss you I will call again Tuesday afternoon.

Joe Cox

I hope

Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027
Tel: 303-735-2296/ FAX: 303-492-8731

August 2, 1998

H. Franklin Bunn, M.D.
Hematology/Oncology Division: LMRC 2
Brigham and Women's Hospital, Room 223
221 Longwood Avenue
Boston, MA 02115
FAX: 617-739-0748

Dear Dr. Bunn,

We spoke several times about a year ago concerning a grant I was submitting on EPO. I was interested in obtaining the UT-7/EPO cell line from you. I received the grant and am getting ready to begin in vitro assays. As you requested, I contacted Dr. Komatsu and received his permission to obtain the UT-7/EPO cell line from you (see attached e-mail letter). I will be glad to reimburse you for shipping expenses. Before you send the cell line, would you please FAX or e-mail me the media and supplements you use to propagate the cell line. I couldn't find this information in your papers.

Our shipping address is: Bolder Biotechnology, Inc.
Porter Biosciences Building
Room 0058; Attention Mike Pettit
University of Colorado
Boulder. CO 80309

Thanks for your help.

Sincerely yours,

Joe Cox, Ph.D.
e-mail: BolderBio@aol.com

Subj: UT-7/EPO cell line
Date: 98-06-12 00:05:16 EDT
From: nkomatsu@ms.jichi.ac.jp (Norio Komatsu)
To: BolderBio@aol.com

Dr. Joe Cox, Ph.D.
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027
USA

Dear Dr. Cox:

Thank you for your interest in my cell line UT-7/EPO.
Please use UT-7/EPO cell line for the purpose you described in your letter.
Please do not allow this cell line to leave your laboratory without prior consent from me.
If the cells were to be for commercial purposes, please contact me.
If you have any question, please give me a letter by e-mail.

Sincerely yours,

Norio Komatsu, M.D., Ph.D.
Division of Hematology
Department of Medicine
Jichi Medical School
Minamikawachi-machi
Tochigi 329-04, JAPAN
FAX: 81-285-44-5258
E-MAIL: nkomatsu@ms.jichi.ac.jp

———————— Headers ————————
Return-Path: <nkomatsu@ms.jichi.ac.jp>
Received: from relay16.mx.aol.com (relay16.mail.aol.com [172.31.106.72]) by air16.mail.aol.com (v43.25) with SMTP; Fri, 12 Jun 1998 00:05:16 -0400
Received: from ms.jichi.ac.jp (ms.jichi.ac.jp [202.233.240.13])
by relay16.mx.aol.com (8.8.5/8.8.5/AOL-4.0.0)
with ESMTP id AAA18353 for <BolderBio@aol.com>;
Fri, 12 Jun 1998 00:03:55 -0400 (EDT)
Received: from [202.233.243.52] ([202.233.243.52])
by ms.jichi.ac.jp (8.8.7/3.6Wbeta7) with SMTP id MAA17828
for <BolderBio@aol.com>; Fri, 12 Jun 1998 12:54:22 +0900 (JST)
Message-Id: <199806120354.MAA17828@ms.jichi.ac.jp>
X-Mailer: Macintosh Eudora Pro Version 2.1.4-J
Mime-Version: 1.0
Content-Type: text/plain; charset="ISO-2022-JP"
Date: Fri, 12 Jun 1998 13:29:57 +0800
To: BolderBio@aol.com
From: nkomatsu@ms.jichi.ac.jp (Norio Komatsu)
Subject: UT-7/EPO cell line

EXHIBIT E

Rev. 013098a
08/21/98

INVOICE

American Type Culture Collection

10801 University Boulevard
Manassas, Virginia 20110-2209 USA
703-365-2700 FAX: 703-365-2750
INTERNET: sales@atcc.org

DETACH AND RETURN TOP PORTION OF THIS INVOICE WITH YOUR PAYMENT.
FOR PAYMENTS BY CREDIT CARD, FILL OUT THE REVERSE SIDE AND RETURN.

To insure proper credit to your account, please provide your

ATCC ACCOUNT#: _____

AMOUNT PAID: $ _____

INVOICE NUMBER/DATE
IV39736 - 39809
08/21/98

BILL-TO:

39809
Bolder Biotechology, Inc.
678 West Willow Street
Louisville, CO 80027
USA

P.O. 074

SHIP-TO:

00046716
Bolder Biotechology, Inc.
Porter Biosciences, Room 0058
University of Colorado
Boulder, CO 80309
USA

Due 30 days from invoice

SALES ORDER #:	SO44275	INVOICE #:	IV39736	PO #: 074	**SHIPPED VIA: Airborne**
ORDER DATE:	08/19/98	INVOICE DATE:	08/21/98	FOB: Manassas, VA	**SHIP DATE: 08/20/98**
SALESPERSON:	ntumba	SOLD TO:	39809	BOL:	

#	ITEM NO	UM	QTY SHIP	QTY B.O.	TAX	DESCRIPTION	PRICE	NET PRICE
001	CRL-2003	EA	1.00		No	TF-1 HUMAN, BONE MARROW, ERYTHROLEUKEMIA	155.00	155.00

Taxable Total: 0.00

Tax[1]: 00.00% Tax[2]: 00.00% Tax[3]: 00.00%

Amount Pre-paid: 0.00

Line Total:	**$155.00**
Total Tax:	**0.00**
Shipping & Handling	**$33.75**
Discount 00.00%	
USD Grand Total:	**$188.75**

ATTENTION!
Our new remittance address is
ATCC, PO Box 3605, Manassas, VA 20110

IMPORTANT --
On orders, inquiries, & payments, refer to this assigned ACCOUNT # **39809**

R&D SYSTEMS INVOICE

Invoice No.: 841827-T

Order Taken by: amandal
Sales Rep.: 703
FOB: Origin
Shipped: 08/26/98
Pack Slip: 980826-05 0002
R&D Order: BT-0194618

Ship via: FX1 - Federal Express
P.O.: 087

Ship to:
Attn: MIKE PETTIT
BOLDER BIOTECHNOLOGY, INC.
PORTER BIOSCIENCES, ROOM 0058
UNIVERSITY OF COLORADO
BOULDER, CO 80309

Bill to:
Attn: GEORGE COX
BOLDER BIOTECHNOLOGY, INC.
678 WEST WILLOW STREET
LOUISVILLE, CO 80027

No: 841827-T

Customer P.O.: 087
COM1:
COM2:

	Qty Ord:	Qty Shp:	Packaging Size/Unit:	Catalog Number: Lot:	Description:	Unit Price:	Extended Price:
1.	1	1	500 UN	287-TC FI078041	Recombinant Human EPO (Tissue Culture Grade), 500 un	$ 395.00	$ 395.00
2.	1	1	5 UG	215-GM-005 AR058032	Recombinant Human GM-CSF	$ 205.00	$ 205.00
					Shipping/Handling Charge		$ 25.00
					Total (U.S. Dollars):		$ 625.00

Account No.: 40348-9
Invoice Date: 08/26/98

Invoice No.: 841827-T
Terms: Net 30 Days
Amount: (U.S. $) $ 625.00

Customer P.O.: 087

Tax ID:
Purch. Agent: Joe Cox
(303) 735-2296

Remit To: R&D Systems, Inc.
Accounts Receivable
614 McKinley Place NE
Minneapolis, MN 55413-2647

Phone: 612-379-2956
TIN: 41-1280894

Notice -- On July 1, 1998, R&D Systems (TECHNE) bought Genzyme's research reagents business. As of August 1, 1998 all ordering (1-800-343-7475) and invoicing will be done through R&D Systems.

Please remit your payment to the above address:

Rev. 013098a
09/25/98

INVOICE

American Type Culture Collection

10801 University Boulevard
Manassas, Virginia 20110-2209 USA
703-365-2700 FAX: 703-365-2750
INTERNET: sales@atcc.org

DETACH AND RETURN TOP PORTION OF THIS INVOICE WITH YOUR PAYMENT.
FOR PAYMENTS BY CREDIT CARD, FILL OUT THE REVERSE SIDE AND RETURN.

To insure proper credit to your account, please provide your

ATCC ACCOUNT#: _____

AMOUNT PAID: $ _____

INVOICE NUMBER/DATE
IV45144 - 39809
09/25/98

BILL-TO:

39809
Bolder Biotechology, Inc.
678 West Willow Street
Louisville, CO 80027
USA

P.O. 100

SHIP-TO:

00068054
Bolder Biotechology, Inc.
University of Colorado
Porter Biosciences
Rm. 0058
Bolder, CO 80309
USA

Due 30 days from invoice

SALES ORDER #:	so50133	INVOICE #:	IV45144	PO #:	100	**SHIPPED VIA:**	**Airborne**
ORDER DATE:	09/21/98	INVOICE DATE:	09/25/98	FOB:	Manassas, VA	**SHIP DATE:**	**09/24/98**
SALESPERSON:	100	SOLD TO:	39809	BOL:			

#	ITEM NO	UM	QTY SHIP	QTY B.O.	TAX	DESCRIPTION	PRICE	NET PRICE
001	HTB-9	EA	1.00		No	5637 CARCINOMA, BLADDER, PRIMARY, HUMAN	155.00	155.00
002	CRL-9589	EA	1.00		No	AML-193 ACUTE MONOCYTIC LEUKEMIA, HUMAN	155.00	155.00
003	CRL-1650	EA	1.00		No	COS1 SV40 TRANSFORMED, AFRICAN GREEN MONKEY, CERCOPITHECUS	155.00	155.00

Taxable Total: 0.00

Tax[1]: 00.00% Tax[2]: 00.00% Tax[3]: 00.00%

Amount Pre-paid: 0.00

Line Total:	**$465.00**
Total Tax:	**0.00**
Shipping & Handling	**$65.52**
Discount 00.00%	
USD Grand Total:	**$530.52**

ATTENTION!
Our new remittance address is
ATCC, PO Box 3605, Manassas, VA 20110

IMPORTANT --
On orders, inquiries, & payments, refer to this assigned ACCOUNT # **39809**

EXHIBIT F

Bolder Biotechnology, Inc.
678 West Willow Street, Louisville, CO 80027
Tel: 303-735-2296/FAX: 303-492-8731

August 10, 1998

Ephraim Johnson
Grants Management Specialist
Grants Management Branch
Division of Extramural Activities
45 Center Drive MSC 6600
Bethesda, MD 20892-6600

Dear Mr. Johnson,

This letter addresses the issues raised in your letter of July 24, 1998. I am pleased to hear you are considering funding this grant application and hope these issues can be resolved satisfactorily so that we can begin work on this exciting project.

I wish to confirm that as of August 1, 1998 I became a full-time employee (100% time) of Bolder Biotechnology, Inc. At the time this grant was submitted I was a part-time employee (60% time) of Bolder Biotechnology. The change to 100% time with Bolder Biotechnology, Inc. is permanent.

A listing of Other Support for myself and other key personnel, including grants under review and planned for submission, is provided on the next page. Bolder Biotechnology currently has two on-going SBIR grants that list me as the Principal Investigator. These grants relate to creating long-acting forms of Growth Hormone and Erythropoietin using the technique of site-specific PEGylation. One of the grants began in January 1998 and will be completed by the end of October 1998. The second grant began in June 1998 and is expected to be completed by the end of December 1998. If the SBIR grant under review is awarded, I will reduce my time devoted to these other grants during the time they overlap, as detailed on the next page.

In our grant application we propose a fee/profit of $4,000. Combined with direct costs of $86,500 and indirect costs of $9,500, this gives a total cost for the grant of $100,000. We feel this budget is appropriate and do not propose any changes.

Please feel free to contact me if you require additional information relating to any of these grants or other matters.

Sincerely,

George Cox, Ph.D.
President and Chief Scientific Officer

CC: Philip F. Smith, Ph.D.

EXHIBIT G

Project No.

Book No.

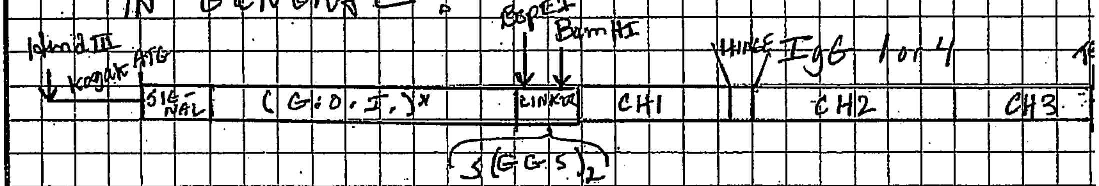
TITLE IGG FUSIONS w GH, EPO,

From Page No.

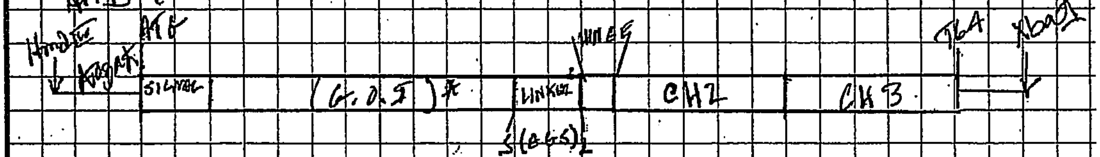
OCTOBER 1, 1998

CONSTRUCT FUSIONS OF (GH, EPO, GCSF) to (N) CH1 OF IgG1 + IgG4 + (N) HINGE REGION IgG1 + IgG4

IN GENERAL:



AND:



*G.O.I. - hu GH, hu EPO, hu G-CSF

Clone fusions in pCDNA3.1(+) (Hind III - Xba cut) (INVITROGEN) FOR EXPRESSION

① PCR IgG1 + IgG4 [CH1 → CH3] + [Hinge → CH3] as Bam - Xba clone + sequence in pCDNA3.1

② PCR EACH G.O.I AS HindIII - BamHI, clone and SEQUENCE IN pCDNA3.1(+)

③ Reject + COMBINE G.O.I's w IgG SEGMENTS THROUGH BamHI SITE -

To Page No

Witnessed & Understood by me, [signature] Date 10/19/98

Invented by [signature] Date 10/19/98

Recorded by

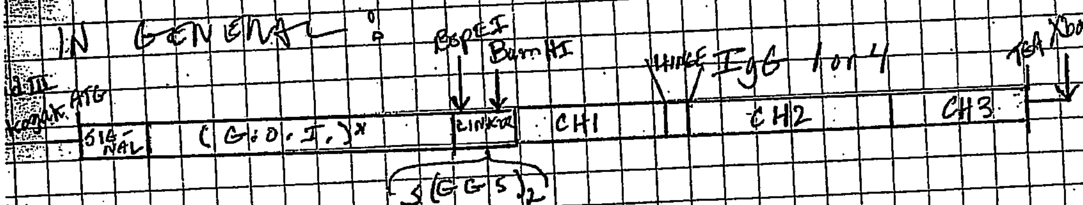
Project No.
Book No.
TITLE IGG FUSIONS w GH, EPO, GCSF

Page No.

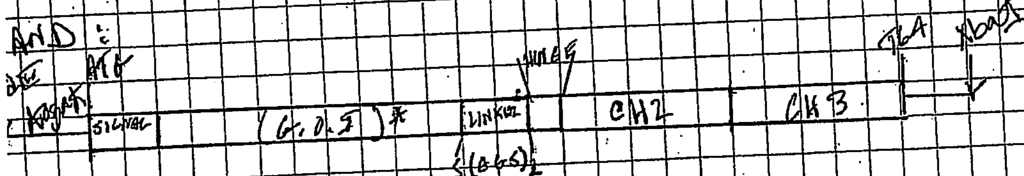
OCTOBER 1, 1998

CONSTRUCT FUSIONS OF (GH, EPO, GCSF) C-term
(A) CH1 OF IgG1 + IgG4 +
(B) HINGE REGION IgG1 + IgG4

IN GENERAL:



AND:



*G.O.I. - hu GH, hu EPO, hu G-CSF

CLONE FUSIONS in pCDNA3.1(+) (INVITROGEN)
(~~HindIII - XbaI cut~~)
FOR EXPRESSION

① PCR IgG1 + IgG4 [CH1 → CH3] + [Hinge → CH3] as Bam - Xba clone + sequence in pCDNA3.1(+)

② PCR EACH G.O.I AS HindIII - BamHI, clone and SEQUENCE IN pCDNA3.1(+)

③ Reset + COMBINE G.O.I's w IgG SEGMENTS THROUGH BamHI SITE -

To Page No. 145

Witnessed & Understood by me, [signature] Date 10/19/98

Invented by [signature]
Recorded by
Date 10/19/98

[redacted]

Project No.______
Book No.______

Page No. 144 OCTOBER 1, 1998

IgG1 SEQUENCE + POSITION OF RELEVANT OLIGOS

OLIGO SEQUENCES GIVEN p 147

```
      81 →
Bam (GG3) GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC
          CGG AGG TGG TTC CCG GGT AGC CAG AAG GGG GAC CGT GGG AGG AGG TTC TCG TGG
           A   S   T   K   G   P   S   V   F   P   L   A   P   S   S   K   S   T

TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG
AGA CCC CCG TGT CGC CGG GAC CCG ACG GAC CAG TTC CTG ATG AAG GGG CTT GGC
 S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F   P   E   P

GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG
CAC TGC CAC AGC ACC TTG AGT CCG CGG GAC TGG TCG CCG CAC GTG TGG AAG GGC
 V   T   V   S   W   N   S   G   A   L   T   S   G   V   H   T   F   P

GCT GTC CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC
CGA CAG GAT GTC AGG AGT CCT GAG ATG AGG GAG TCG TCG CAC CAC TGG CAC GGG
 A   V   L   Q   S   S   G   L   Y   S   L   S   S   V   V   T   V   P

TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC
AGG TCG TCG AAC CCG TGG GTC TGG ATG TAG ACG TTG CAC TTA GTG TTC GGG TCG
 S   S   S   L   G   T   Q   T   Y   I   C   N   V   N   H   K   P   S
                          BamGG5          83 →
AAC ACC AAG GTG GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
TTG TGG TTC CAC CTG TTC TTT CAA CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
 N   T   K   V   D   K   K   V   E   P   K   S   C   D   K   T   H   T

TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC
ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG
 C   P   P   C   P   A   P   E   L   L   G   G   P   S   V   F   L   F  104
                  102
CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC
GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG
 P   P   K   P   K   D   N   L   M   I   S   R   T   P   E   V   T   C

GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG
CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC
 V   V   V   D   V   S   H   E   D   P   E   V   K   F   N   W   Y   V

GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG
 D   G   V   E   V   H   N   A   K   T   K   P   R   E   E   Q   Y   N

AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA
 S   T   Y   R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N

GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC
 G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A   P   I   E

AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG
TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC
 K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y   T   L
                                                           103 →
CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG
 P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V
                                                       105
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG
TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC
 K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P

GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG
 E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F

CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC
GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG
 L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G   N   V   F

TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC
AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG
 S   C   S   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S   L

TCC CTG TCT CCG GGT AAA TGA gtgcg acggccggca agcccccgct ccccgggctc
AGG GAC AGA GGC CCA TTT ACT cacgc tgccggccgt tcgggggcga ggggcccgag
 S   L   S   P   G   K   *

tcgcggtcgc acgaggatgc ttggcacgta ccccgtgtac atacttcccg ggcgcccagc
agcgccagcg tgctcctacg aaccgtgcat ggggcacatg tatgaagggc ccgcgggtcg
            ← 82                          Xba
```

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Read & Understood by me, Date 10/19/98 | Invented by | Date 10/19/98 | Recorded by

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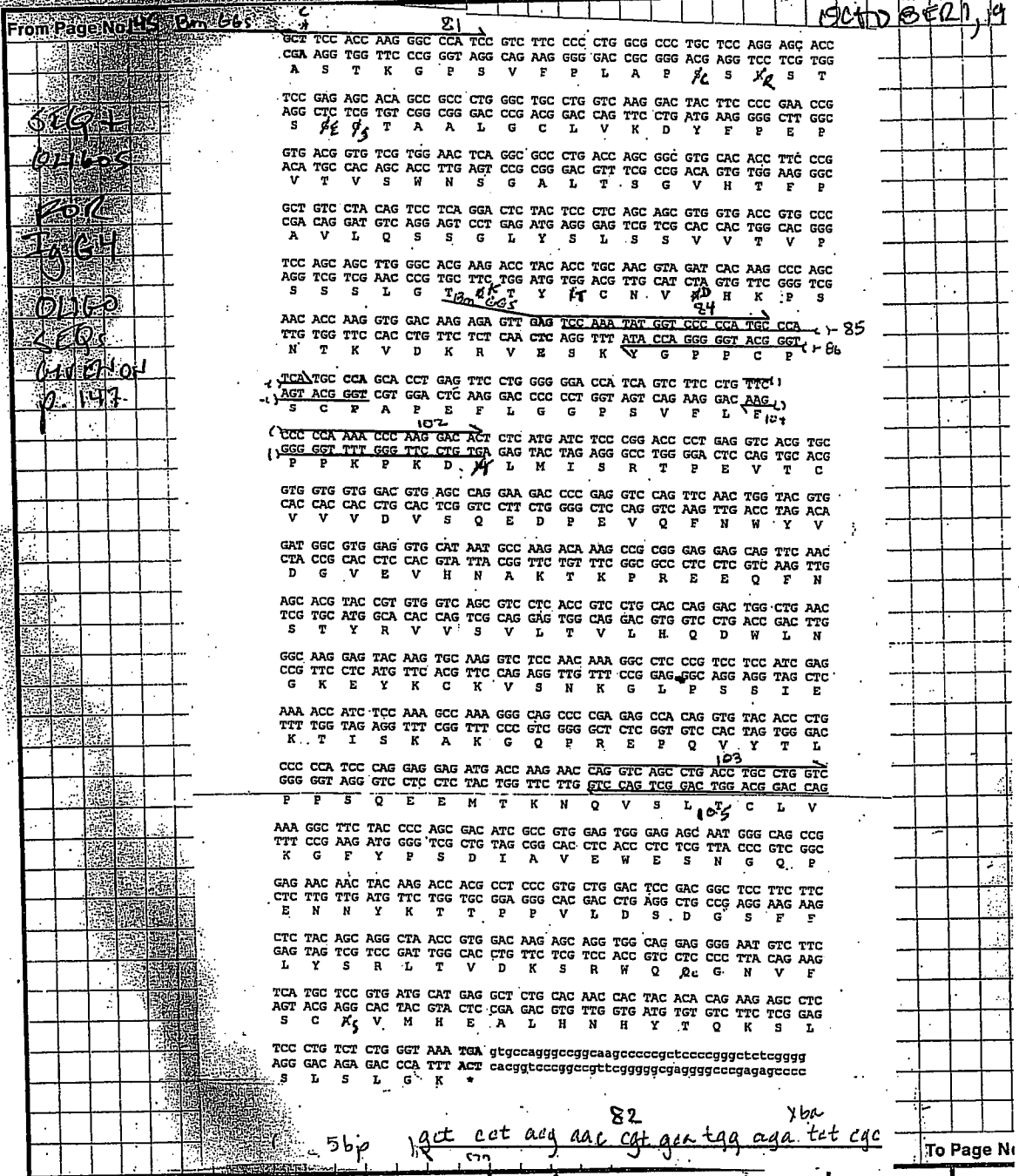


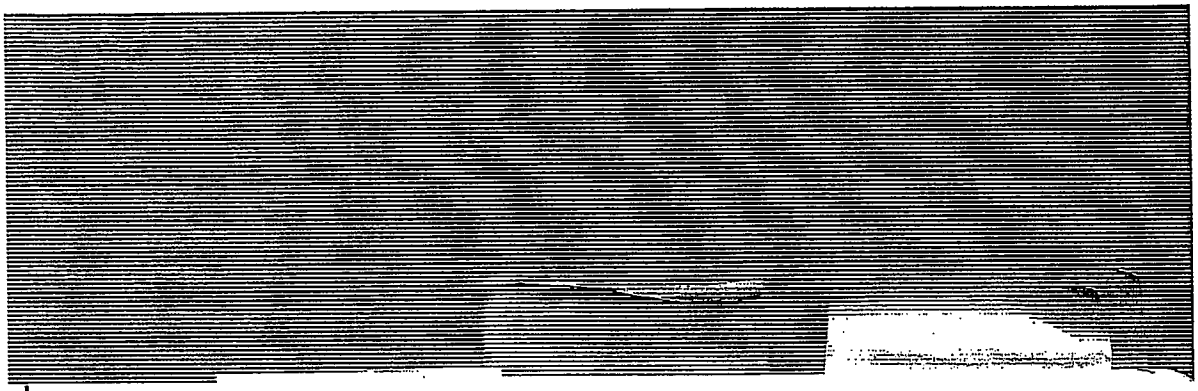
Witnessed & Understood by me:

Date 10/14/98

Invented by

Recorded by

Date 10/19/98



Project No.

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m Page No. 147

OCTOBER 1, 1998

IgG 1/4 OLIGOS

BB81 (IgG1/4 CH1 forward) 5>CGC GGA TCC GGT GGC TCA GCC TCC ACC AAG GGC CCA TC>3

BB82 (IgG1/4 3'UTR rev) 5> CGC TCT AGA GGT ACG TGC CAA GCA TCC TCG >3

BB83 (IgG1 HINGE forward) 5>CGC GGA TCC GGT GGC TCA GAG CCC AAA TCT TGT GAC AAA ACT>3

BB84 (IgG4 HINGE forward) 5> CGC GGA TCC GGT GGC TCA GAG TCC AAA TAT GGT CCC CCA TGC>3

BB85 (IgG4 HINGE forward) 5> TCC AAA TAT GGT CCC CCA TGC CCA TCA >3

BB86 (IgG4 HINGE reverse) 5> TGG GCA TGA TGG GCA TGG GGG ACC ATA >3

BB87(GH IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GCT ACA GGC TCC CGG ACG >3

BB102 (IGG1/4 SEQ forward in CH2) 5>TTC CCC CCA AAA CCC AAG GAC AC>3

BB103 (IGG1/4 SEQ forward in CH3) 5>CAG GTC AGC CTG ACC TGC CTG GTC>3

BB104 (IGG1/4 SEQ reverse in CH2, complement of BB102) 5>GT GTC CTT GGG TTT TGG GGG GAA>3

BB105 (IGG1/4 SEQ reverse in CH3, complement of BB103) 5>GAC CAG GCA GGT CAG GCT GAC CTG>3

SET UP PCRs FOR:

IgG1 "CH1" FULL LENGTH CH1, Hinge, CH2, 3

& "Hinge" Hinge, CH_2 CH_3

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OCTOBER 1998

PCRS: TEMPLATE = 1 µl, 1 ng CLONTECH HPB Leukocyte cDNA, CAT# 7182-1

"CH1" #1		"Hinge" #2	
1 µl	TEMPLATE	1 µl	TEMPLATE
10 µl	10x PCR Buffer (PROMEGA)	10 µl	10x PCR Buffer (PRO)
10 µl	10x dNTPs	10 µl	10x dNTPs
6 µl	25mM $MgCl_2$	6 µl	25mM $MgCl_2$
2.5 µl	BB81 (10 pmol/µl)	2.5 µl	BB83 (10 pmol/µl)
2.5 µl	BB82 (10 pmol/µl)	2.5 µl	BB82 "
0.3 µl	Taq Pol (PRO)	0.3 µl	Taq Pol (PRO)
0.1 µl	Pfu Pol (STRAT)	0.1 µl	Pfu Pol (STRAT)
67.6 µl	H_2O	67.6 µl	H_2O

~~MAKE 2X EACH RXN, = 200 µl~~ DH2O

~~100 µl x 2~~ DH2O

SPLIT ~~200 µl → 66 µl x 3~~ + ANNEAL @ 3 temps

100 µl → 50 µl x 2 DH2O

CYCLE: 95°C 3 MIN

33 cycles { 95°C 1 MIN; 58°C or 66°C 1 MIN; 72°C 1 1/2 MIN }

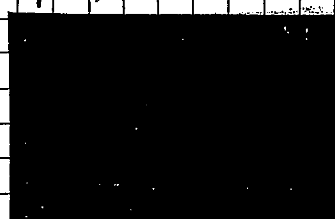
6°C HOLD

~2:35 → 4:45

RUN OUT: 1.2% Ag SK, 1X TBE

125V
START – 4:50
STOP – 5:30

1 λ/H + ΦX
2 –
3 PCR-1 (58°C) 5/50 µl
4 " (66°C) "
5 –
6 PCR-2 (58°) 5/50
7 (66°) "
8
9 λ/H + ΦX



LOOKS GOOD –
SIZES ALL CONSISTENT WITH EXPECTED –
BOTH ANNEALING TEMPS SEEM TO WORK!

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Witnessed & Understood by me,	Date	Invented by / Recorded by	Date
[signature]	10/19/98	[signature]	10/19/98

TLE________ Project No.______ Book No.______

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OCTOBER 2, 1998

CLEAN UP PCRs & Pool Both temp Runs -

Clean up w Qia spin PCR clean up - Elute 50 ul EB

go to digests c̄ BamHI + XbaI

PCR ①	PCR ②	pCDNA3.1(-) (3/23/98)
~45 ul PCR ①	~45 ul PCR ②	7 ul QSP ~2 ug
40 ul H_2O	40 ul H_2O	81 ul H_2O
10 ul NEB2 (10X)	10 ul NEB2 (10X)	10 ul 10x NEB2
1 ul 100x BSA	1 ul 100x BSA	1 ul 100x BSA
2 ul BamHI 40u	2 ul BamHI 40u	0.5 ul BamHI 10u
2 ul XbaI 40u	2 ul XbaI 40u	0.5 ul XbaI 10u

All @ 37°C ~ 10:40

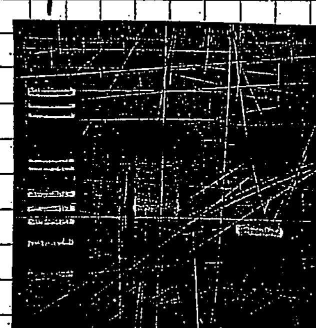
@ 12:10 ADD 1 ul CIP (10u) NEB TO pCDNA only

@ 1:10 - EtOH Precip, wash Pellet w 70% EtOH

AIR DRY ~37°C ~30 MIN Resus + Run out

PREP GEL PRCS: 1.2% Ag ~~Gel~~ 1X TAE START - 2:15 STOP - 3:30

1 ΦX
2 PCR① x Bam+Xba
3 -
4 PCR② x Bam+Xba
5



XCISE BANDS
Elute in QIAEX II

Elute Resin
w 25 ul EB
pull 20 ul
STORE @ -20°C
o/n

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Book No.______ TITLE ______

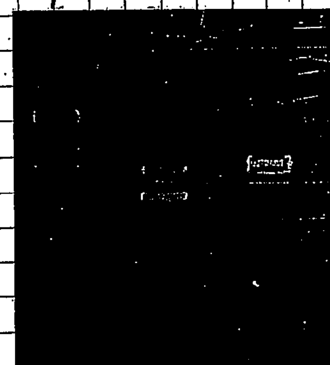
From Page No. 149

OCTOBER 2, 1998

pCDNA3.1(+) Prep Gel: 0.6% Ag 5X 1X TAE

START - 2:15
STOP - 3:30

LANE
1 λ/H
2 pCDNA3.1(+) NOT (1.5X)
3 pCDNA3.1(+) X(Bam+Xba+CIP)



WELL SEPARATED
linear vs UNCUT

BUT MAYBE SOME
DEGRADATION OF
Bam-Xba VECTOR!?

XCISE VECTOR BAND

Elute (QIAEX II) Elute Resin
with 25ul TE, pull 20ul) STORE @ -20C

10/4 SET UP LIGATIONS: 10/4

Lig ①	Lig ②	Lig ③
2ul pCDNA3.1(BXCip) g-p	2ul pCDNA3.1(BXCip) g-p	2ul pCDNA3.1(BXC…
2ul PLRD(B-X) g-p	2ul pCR② (BX) g-p	2ul H_2O
3.5ul H_2O	3.5ul H_2O	3.5ul H_2O
2ul 5X Lig Buffer (BRL)	2ul 5X Lig Buffer BRL	2ul 5X Lig Buffer
0.5ul T4 ligase 1U/ul (BRL)	0.5ul T4 ligase	0.5ul T4 Ligase

@ 15°C ON ~ 2:30p 10/4

10/5 PULL Ligs + go to Tfm p151

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[redacted]

[redacted]

Project No.____
.E____ Book No.____

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OCTOBER 5, 1998

Transform with lig 1, 2, 3 (10/4)

T=0 5ul each lig, 5ul BLANK ϕ DNA + 50ul comp DH5α

HOLD ON ICE 10min, → 42°C for 45 sec,

CHILL ON ICE, dil 1/10 w SOC, in 37°C ~60min

PLATE OUT LB Ap100 10ul, 100ul each

@ 37°C ~5:15p

10/6 PULL PLATES, LOOKS GOOD -

(CHI) lig ① 10ul ; 11 cfu
100ul ; ~(100-200) LOOKS GOOD

(Hinge) lig ② 10ul ; 250
100ul ; ~500

PICK 4 ISOLATED COLS FROM #1, #2
INOC 4.5ul LB Ap100 @ 37°C ON

lig ③ 10ul ; ϕ
100ul ; 8 cfu

SAVE PLATES @ 4°C

BLNK 10ul ; ϕ
100ul ; ϕ

10/7 ONs UP OK 1-(1→4) 2-(1→4) → STREAK FOR SINGLES ON LB Ap100

QSP [2ml/EPP] ×2 elute each 50ul EB, POOL Σ ~100ul

OD's (dil 1/100 A_{260})

	A_{260}	conc		A_{260}	conc
1-1	.106	0.53 µg/ul	2-1	~~.106~~ .091	0.46 µg/ul
1-2	.157	0.79 "	2-2	.107	0.54 "
1-3	.150	0.75 "	2-3	.128	0.64 "
1-4	.117	0.59	2-4	.084	0.42 "

cut + run

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Recorded by

From Page No. 151

OCTOBER 8, 1998

Digest + run out to check inserts –

	x BamHI		x BamHI + XbaI
10x	~3 ul QBP	10x	~3 ul QBP
60	6 ul 10x NEB2	50	5 ul 10x NEB2
420	42 ul H2O	410	41 ul H2O
5	0.5 ul BamHI (10u)	5	0.5 ul Bam (10u)
		5	0.5 ul XbaI (10u)

MAKE UP 10X RXN MIX, ALIQUOT 47 ul TO TUBES WITH VARYING AMT OF PLASMID ≅ 2 ug

1-1 : 4 ul	2-1 : 4 ul	pCDNA3.1(+) : 7 ul
1-2 : 2.5 ul	2-2 : 4 ul	
1-3 : 2.5 ul	2-3 : 3.3 ul	
1-4 : 3.3 ul	2-4 : 5 ul	

ALL DIGESTS @ 37°C ~10:00 → 11:30

RAN OUT 10 ul/50 ul Digests. 0.7% Ag SK 1X TAE

GEL ① — ① 75V START – 11:4? STOP – 1:00

LANE	GEL ①	LANE	GEL ②
1	λH + φX ; pCDNA x Bam 10/50	1	λH + φX
2	1-1	2	pCDNA x Bam x Xba 10/50
3	1-2	3	1-1
4	1-3	4	1-2
5	1-4	5	1-3
6	2-1	6	1-4
7	2-2	7	2-1
8	2-3	8	2-2
9	2-4	9	2-3
10		10	2-4

② 75V START – 11:55 STOP – 1:00

PHOTOS p 169

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Invented by [signature] Recorded by Date 10/14/98

[redacted]

[redacted]

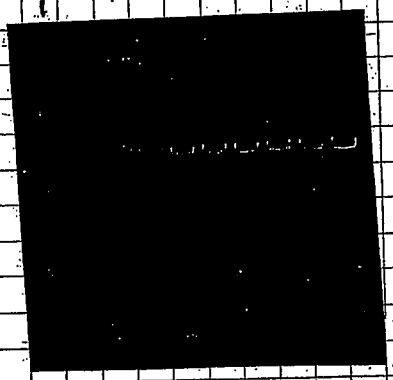
Project No.

LE Book No. 167

m Page No. 164

OCTOBER 8 1998

(1)



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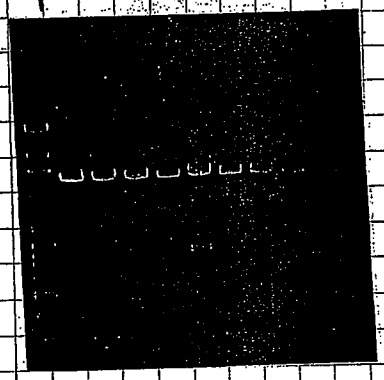

ALL LOOK OK, INSERTS CORRECT SIZE, ONLY ONE INSERT + QUANTITY CONSISTENT WITH OD -

SEND OFF TO MMR FOR SEQ. 10/8/98

TEMPLATES 10/8/98

#		CONC (ug/ul)	VOL (ul)
QS #	1-1 (10/7/98)	0.53	40
"	1-2 "	0.79	40
"	1-3 "	0.75	40
"	1-4 "	0.59	40
"	2-1 "	0.46	40
"	2-2 "	0.54	40
"	2-3 "	0.64	40
"	2-4 "	0.42	40

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Witnessed & Understood by me, [redacted]

Date 10/19/98

Invented by [redacted]

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Date 10/19/98

168 Project No. ______ Book No. ______ TITLE CLONING EPO + GH FOR IGG FUS

From Page No. 164, 163

OCTOBER 8, 1998

PCR OUT EPO + GH WITH C-TERMINAL SEQS. FOR FUSION VIA RECOMBINATION WITH IgG CLONED AT LINKER S(GGS)2

GROWTH HORMONE SEQ + OLIGOS

hGH w SIGNAL

```
       87 →
ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCTGCCCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATT
 M  A  T  G  S  R  T  S  L  L  L  A  F  G  L  L  C  L  P  W  L  Q  E  G  S  A  F  P  T  I

CCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTGGCCTTTGACACCTACCAGGAGTTTGAAGAAGCC
 P  L  S  R  L  F  D  N  A  M  L  R  A  H  R  L  H  Q  L  A  F  D  T  Y  Q  E  F  E  E  A

TATATCCCAAAGGAACAGAAGTATTCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACAGG
 Y  I  P  K  E  Q  K  Y  S  F  L  Q  N  P  Q  T  S  L  C  F  S  E  S  I  P  T  P  S  N  R
                         43 →
GAGGAAACACAACAGAAATCCAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAGG
 E  E  T  Q  Q  K  S  N  L  E  L  L  R  I  S  L  L  L  I  Q  S  W  L  E  P  V  Q  F  L  R

AGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCTAGAGGAAGGCATCCAAACGCTG
 S  V  F  A  N  S  L  V  Y  G  A  S  D  S  N  V  Y  D  L  L  K  D  L  E  E  G  I  Q  T  L
                                             ← CGTTGCAGATACTGGAGG 9
ATGGGGAGGCTGGAAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAAACTCACACAACGATGAC
 M  G  R  L  E  D  G  S  P  R  T  G  Q  I  F  K  Q  T  Y  S  K  F  D  T  N  S  H  N  D  D

GCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGACATGGACAAGGTCGAGACATTCCTGCGCATCGTGCAGTGCCGCTCT
 A  L  L  K  N  Y  G  L  L  Y  C  F  R  K  D  M  D  K  V  E  T  F  L  R  I  V  Q  C  R  S

GTGGAGGGCAGCTGTGGCTTCTAGCTGCCCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCA
 V  E  G  S  C  G  F  *     G  G  S
← CACCTCCCGTCGACACCGAAG AGG CCT A AGG CGC
       88                 CCT
```

9. BB9 (GHR SEQ) 18 5′ GGAGGTCATAGACGTTGC 3′

43. BB43 (GH SEQ corrected BB8) 5′ TCCAACCTAGAGCTGCTC 3′

87. BB87(GH IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GCT ACA GGC TCC CGG ACG >3

88. BB88 (GH IGGFUS rev) 5> CGC GGA TCC TCC GGA GAA GCC ACA GCT GCC CTC CAC >3

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[redacted]

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October 8, 1998

EPO SEQ + OLIGOS

```
            H3 kc3           89
GGATCC ATG GGG GTG CAC GAA TGT CCT GCC TGG CTG TGG CTT CTC CTG TCC CTG CTG
CCTAGG TAC CCC CAC GTG CTT ACA GGA CGG ACC GAC ACC GAA GAG GAC AGG GAC GAC
        M   G   V   H   E   C   P   A   W   L   W   L   L   L   S   L   L

TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG
AGC GAG GGA GAC CCG GAG GGT CAG GAC CCG CGG GGT GGT GCG GAG TAG ACA CTG TCG GCT CAG GAC
 S   L   P   L   G   L   P   V   L   G   A   P   P   R   L   I   C   D   S   R   V   L

GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT ATC ACG ACG GGC TGT GCT GAA CAC TGC AGC
CTC TCC ATG GAG AAC CTC CGG TTC CTC CGG CTC TTA TAG TGC TGC CCG ACA CGA CTT GTG ACG TCG
 E   R   Y   L   L   E   A   K   E   A   E   N   I   T   T   G   C   A   E   H   C   S

TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC TAT GCC TGG AAG AGG ATG GAG GTC
AAC TTA CTC TTA TAG TGA CAG GGT CTG TGG TTT CAA TTA AAG ATA CGG ACC TTC TCC TAC CTC CAG
 L   N   E   N   I   T   V   P   D   T   K   V   N   F   Y   A   W   K   R   M   E   V

                54
GGG CAG CAG GCC GTA GAA GTC TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT GTC CTG CGG GGC CAG
CCC GTC GTC CGG CAT CTT CAG ACC GTC CCG GAC CGG GAC GAC AGC CTT CGA CAG GAC GCC CCG GTC
 G   Q   Q   A   V   E   V   W   Q   G   L   A   L   L   S   E   A   V   L   R   G   Q

GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG CAG CTG CAT GTG GAT AAA GCC GTC AGT
CGG GAC AAC CAG TTG AGA AGG GTC GGC ACC CTC GGG GAC GTC GAC GTA CAC CTA TTT CGG CAG TCA
 A   L   L   V   N   S   S   Q   P   W   E   P   L   Q   L   H   V   D   K   A   V   S
                                                                      55

GGC CTT CGC AGC CTC ACC ACT CTG CTT CGG GCT CTG GGA GCC CAG AAG GAA GCC ATC TCC CCT CCA
CCG GAA GCG TCG GAG TGG TGA GAC GAA GCC CGA GAC CCT CGG GTC TTC CTT CGG TAG AGG GGA GGT
 G   L   R   S   L   T   T   L   L   R   A   L   G   A   Q   K   E   A   I   S   P   P

GAT GCG GCC TCA GCT GCT CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC CGA GTC
CTA CGC CGG AGT CGA CGA GGT GAG GCT TGT TAG TGA CGA CTG TGA AAG GCG TTT GAG AAG GCT CAG
 D   A   A   S   A   A   P   L   R   T   I   T   A   D   T   F   R   K   L   F   R   V

TAC TCC AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG GAC AGA
ATG AGG TTA AAG GAG GCC CCT TTC GAC TTC GAC ATG TGT CCC CTC CGG ACG TCC TGT CCC CTG TCT
 Y   S   N   F   L   R   G   K   L   K   L   Y   T   G   E   A   C   R   T   G   D   R
                                                            90                    S G G
                                                                                     Bn
TGA CCAGGTGTCCACCTGAGCATAACTACCAGAATTC
ACT GGTCCACAGGTGGACTCGTATTGATGGTCTTAAG
 *
```

54. BB54 (epo sq forward) 5> CAGCAGGCCGTAGAAGTC >3

55. BB55 (epo sq reverse) 5> ACTGACGGCTTTATCCAC >3

89. BB89 (EPO IGGFUS forward) 5> CGC AAG CTT GCC ACC ATG GGG GTG CAC GAA TGT CCT >3

90. BB90 (EPO IGGFUS reverse) 5> CGC GGA TCC TCC GGA TCT GTC CCC TGT CCT GCA GGC >3

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Witnessed & Understood by me, [signature] Date 10/19/98

Invented by [signature] Date 10/19/98

Recorded by

Project No.______
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From Page No. 129

OCTOBER 8, 1998

PCRs for EPO & GH CLONING FOR IgG FUSION

GH		EPO	
20 ul	10x PCR buffer (PRO)	20 ul	10x PCR buffer PRO
20 ul	10x dNTPs (2mM)	20 ul	10x dNTPs
12 ul	25mM $MgCl_2$	12 ul	25mM $MgCl_2$
138.5 ul	H_2O	137.5 ul	H_2O
4 ul	BB87 (40pmol) p168	4 ul	BB 89 (40 pmol) p168
4 ul	BB88 (") "	4 ul	BB 90 (") "
1 ul	Taq Pol (5u) (PRO)	1 ul	Taq Pol (5u) (PRO)
0.5 ul	Pfu Pol (1.25u) (STRAT)	0.5 ul	Pfu Pol (1.25u) (STRAT)
1 ul	cDNA Hu pituitary (1ng) (CLONTECH)	1 ul	pBBT131 (1ng) → pUC19::EPO (EID)

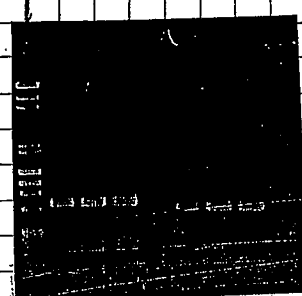
SPLIT INTO 3x66ul | SPLIT INTO 3x66ul

CYCLE:
96°C 3MIN
95°C 1MIN ⎤
(56, 61, 66)°C 1½ MIN ⎥ 30x
72°C 1MIN ⎦
6°C HOLD

START - 1:35
DONE ~ 3:30

RUN OUT: 1.2% Ag SK 1x TAE 80V START - 3:35
STOP - 4:35

LANE			
1	λ/H + ΦX		
2	GH (87/88)	(56°)	6/66ul
3	" "	(61°)	"
4	" "	(66°)	"
5	—		
6	EPO (89/90)	(56°)	6/66ul
7	" "	(61°)	"
8	" "	(66°)	"



BOTH LOOK GOOD

STORE RXNS ON @ -20°C

To Page No. 171

Witnessed & Understood by me, [signature] Date 10/19/98
Invented by [signature] Date 10/19/98
Recorded by

[redacted]

Project No.________
TITLE________________________________ Book No.________

From Page No. 120,

OCTOBER 9, 1998

USE 66°C RXNS (6H + EPO) FOR CLONING;

Clean up WITH Qiagen PCR PURIF; Elute 90ul

Digest VS BamH I + HindIII

~85ul each PCR
10ul 10x NEB2
1ul 100x BSA
2ul BamHI (40U)
2ul HindIII (40U)

@37°C ~9:25
↓
11:55

EtOH PRECIP –
WASH PELLET W
70% EtOH

AIR DRY 37°C ~20 min

RESUS 20ul 1X LB

+ min dup
PREP GEL

(VECTOR)
Digest pCDNA3.1(+)
FOR CLONING

15ul pCDNA3.1(+) (6
73ul H2O
10ul 10x NEB2
1ul BamHI (20
1ul HindIII (2

@ 37°C ~9:25
→ 10:25
ADD 1ul CIP (10U) [illegible]
Let go @ 37°C

11:25 PULL DIG
CLEAN UP WITH
Qiagen PCR PURIF. KIT

Elute 60ul EB +
15ul 5x LB

+ min out Prep gel

To Page No. 12

Witnessed & Understood by me, [signature] Date 10/19/98

Invented by [signature] Date 10/19/98

Recorded by

Project No.

2 Book No. TITLE

From Page No. 91, 145 OCTOBER 9, 1998

PREP GELS (GH, EPO, GCSF) PCR FRAGS FOR

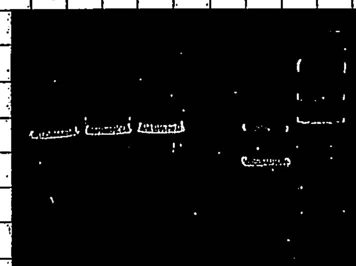
FUSION TO IgG's + pCDNA3.1(+) VECTOR

0.7% Ag SK 1X TAE 100 V START - 12:15
STOP - 1:30

LANE

1 pCDNA3.1(+) Bam+HindIII+CIP
2 " " "
3 " " "
4 —
5 pCDNA3.1(+) NAT 1 ug
6 λ/H

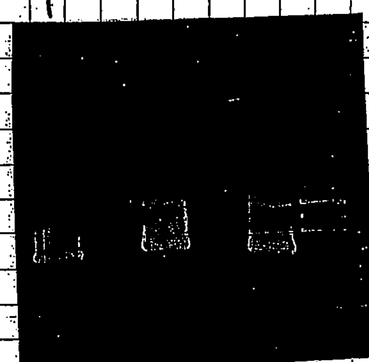
START ? ACTIVITY?



1.2% Ag SK 1X TAE 100 V START 12:55
STOP 1:55

LANE

1 EPO PCR 89/90 (66°) x Bam+HindIII
2 —
3 GH PCR 87/88 (66°) x Bam+HindIII
4 —
5 GCSF PCR 91/92 (66°) x Bam+HindIII
6 ΦX



EXCISE + Elute in QIAEX II

pCDNA 2 SLICES ; Elute 25 ul x 2
POOL 20 ul x 2, 40 ul

PCRs - 1 SLICE EACH, Elute 25 ul, pull 20 ul

GO TO Ligation.

To Page No. 93

Witnessed & Understood by me, Joe G | Date 10/19/98 | Invented by [signature] | Recorded by | Date 10/19/98

[redacted]

[redacted]

Project No.______
TLE________________________ Book No.______ 173

om Page No. 172 OCTOBER 9, 1998

Ligations: GH, EP, CS PCRs FOR IG TISLIN8

Ligs 10/9 —
{ 3 ul pCDNA (HindIII + Bam + CIP) gp (p172)
{ 2 ul 5x BRL lig buffer
{ 2.5 ul H_2O
{ 0.5 ul T4 ligase BRL

\+ 2 ul H_2O ≡ lig CON
\+ 2 ul PCR (87/88) × (HindIII + Bam) gp ≡ lig GH
\+ 2 ul " 89/90 × (") gp ≡ " EP
\+ 2 " 91/92 × (") gp ≡ " CS

@ 15°C ~4:30p 10/9

10/11 Tfm ligs 10/9:

4 ul each lig, 4 ul EB (DLMA) + 36 ul Comp DH5α

T=0 ADD cells to DNA ON ICE
=10 min, Heat shock 42°, 45 sec, chill on ice

dil 1/10 w SOC, inc @37°C ~60 min

PLATE: ligs 10 ul, 100 ul LB Ap100
BLANK 100 ul " "

@ 37°C ON — ~3:30p

10/12 Pull plates looks good —
Store ON @ 4°C

To Page No. 174

Witnessed & Understood by me, [signature] Date 10/19/98

Invented by [signature] Recorded by Date 10/19/98

EXHIBIT H

Project No.

LE EPO - IgG Fusions Book No.

From Page No.

Spin-X column to sterilize
BBT 179 - EPO-IgG1 CH1
180 - EPO IgG1 Fc
181 - EPO-IgG4 Fc
pCDNA3.1 control

R+D Epo as control

do 10x dilutions this time to get ~ ... then repeat w/ 3x dilutions

WT

	[Assay]			[Assay ng/ml]
5u -> 500 ml = 10u/ml ÷ 2 =	5 u/ml	5	6	10
50 -> 500	0.5	4	5	4
50 -> 500	0.05	3	4	.4
50 -> 500	0.005	2	3	.04
50 -> 500	0.0005		2	
50 -> 500			1	

COS cell sups w/ 1 ug/ml

			[Final]
30 ml -> 300 ml	5	6	50 ng/ml
50 ml -> 500	4	5	5
50 -> 500	3	4	0.5
50 -> 500	2	3	0.05
50 -> 500	1	2	0.005
~~50 -> 500~~ 50 -> 500		1	0.0005
~~50 -> 500~~			
~~50 -> 500~~			

CT cell = 117 / field => 1.17 x 10^6 / ml
dil 1 ml Epo
wash 3x
rsp 11.7 ml media

To Page No.

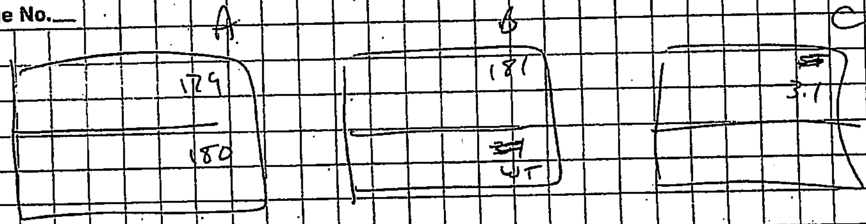
Witnessed & Understood by me, [signature] Date 7/26/99

Invented by [signature] Date 7/12/99

Recorded by

Project No.____
Book No.____ TITLE Epo-IgG Fusion Assay

From Page No.__



T 37 10pm

aliquot 500 Epo - set 9 x 5 ml onto 7; 5 ml short

50 ml cells
50 ml medium [Iscove's DM w/ PR, 10% FBS, Pls; Glu] Fresh

No cells 0 1 2 3 4 5 6

1/15/99 MTS ~ 11:30

Read ~ 2:30 3hr

go to page 95 for data

To Page No.__

Witnessed & Understood by me, [signature] Date 1/26/99

Invented by [signature]
Recorded by

Date 1/12/99

[redacted]

[redacted]

From 8787 Project No.

Epo-IgG Fusions Book No. 95

TEST NO.	:	W/L MODE	: DUAL	DATE	: 01/15/99
TEST NAME	: MTS19498	TEST FILTER	: 490 nm	TIME	: 14:29:42
PLATE	: JC11599A17918	REF. FILTER	: 650 nm	OPERATOR	:

Calculation mode : Endpoint

plate A : 179
180

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.197	0.199	0.193	0.196	0.192	0.196	0.197	0.195	0.196	0.195	0.197	0.195
		no cells	0	1	2	3	4	5	6			
B	0.196	0.241	0.278	0.287	0.297	0.302	0.428	0.996	1.108	0.191	0.191	0.194
C	179 0.194	0.229	0.270	0.289	0.295	0.308	0.422	1.002	1.121	0.192	0.193	0.193
D	0.194	0.238	0.283	0.292	0.298	0.302	0.415	0.997	1.133	0.192	0.190	0.190
E	0.191	0.253	0.272	0.276	0.282	0.306	0.540	1.106	1.128	0.192	0.189	0.193
F	180 0.190	0.247	0.279	0.279	0.279	0.309	0.542	1.099	1.116	0.189	0.189	0.189
G	0.193	0.244	0.262	0.279	0.284	0.305	0.548	1.099	1.121	0.189	0.188	0.191
H	0.187	0.192	0.183	0.187	0.191	0.193	0.190	0.194	0.192	0.189	0.188	0.187

179

no. cells $\bar{x}$ = 0.236
0 $\bar{x}$ = 0.277 − 0.236 = 0.041 +/− 0.007
1 $\bar{x}$ = 0.289 = 0.053 +/− 0.003
2 $\bar{x}$ = 0.297 = 0.061 +/− 0.001
3 $\bar{x}$ = 0.304 = 0.068 +/− 0.003
4 $\bar{x}$ = 0.422 = 0.186 +/− 0.007
5 $\bar{x}$ = 0.998 = 0.762 +/− 0.003
6 $\bar{x}$ = 1.121 = 0.884 +/− 0.013

180

no. cells $\bar{x}$ = 0.248
0 $\bar{x}$ = 0.271 − 0.248 = 0.023 +/− 0.009
1 $\bar{x}$ = 0.278 = 0.030 +/− 0.002
2 $\bar{x}$ = 0.282 = 0.034 +/− 0.003
3 $\bar{x}$ = 0.307 = 0.059 +/− 0.002
4 $\bar{x}$ = 0.543 = 0.295 +/− 0.004
5 $\bar{x}$ = 1.101 = 0.853 +/− 0.004
6 $\bar{x}$ = 1.122 = 0.874 +/− 0.006

To Page No.

Witnessed & understood by me, [redacted] Date 1/26/99

Invented by [redacted] Date 1/15/99

Recorded by [redacted]

Pg 87 11

Project No.______
Book No.______ TITLE Epo Isf Fusions

From Page No.

TEST NO.	:	W/L MODE	: DUAL	DATE	: 01/15/99
TEST NAME	: MTS19498	TEST FILTER	: 490 nm	TIME	: 14:32:27
PLATE	: JC11599B181wt	REF. FILTER	: 650 nm	OPERATOR	:

Calculation mode : Endpoint — Plate B — 181 ~~135A~~ wT epo

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.182	0.193	0.188	0.195	0.187	0.192	0.188	0.190	0.185	0.186	0.196	0.198
(cells)		No cells	0	1	2	3	4	5	6			
B	0.189	0.239	0.285	0.288	0.305	0.340	0.748	1.208	1.236	0.194	0.194	0.194
C (181)	0.185	0.241	0.279	0.298	0.304	0.343	0.737	1.212	1.264	0.192	0.193	0.193
D	0.183	0.248	0.280	0.297	0.303	0.337	0.724	1.280	1.234	0.192	0.196	0.142
E	0.182	0.244	0.284	0.281	0.300	0.341	0.685	1.186	1.242	0.195	0.195	0.193
F (~~135A~~)	0.177	0.244	0.271	0.286	0.293	0.338	0.682	1.180	1.306	0.193	0.182	0.194
G (wT)	0.179	0.246	0.265	0.287	0.298	0.340	0.673	1.187	1.253	0.191	0.191	0.192
H	0.176	0.194	0.181	0.187	0.189	0.191	0.192	0.194	0.190	0.190	0.192	0.190

181

No cells $\bar{x}$ = 0.243

0	$\bar{x}$ = 0.281 − 0.243	= 0.038 +/− 0.003
1	$\bar{x}$ = 0.294	= 0.051 +/− 0.006
2	$\bar{x}$ = 0.304	= 0.061 +/− 0.001
3	$\bar{x}$ = 0.340	= 0.097 +/− 0.003
4	$\bar{x}$ = 0.736	= 0.493 +/− 0.012
5	$\bar{x}$ = 1.210	= 0.967 +/− 0.003
6	$\bar{x}$ = 1.245	= 1.002 +/− 0.017

wT epo

no cells $\bar{x}$ = 0.245

0	$\bar{x}$ = 0.273 − 0.245	= 0.028 +/− 0
1	$\bar{x}$ = 0.285	~~= 0.0917~~ 0.042 +/− 0.
2	$\bar{x}$ = 0.297	= 0.052 +/− 0.0
3	$\bar{x}$ = 0.400	= 0.095 +/− 0.
4	$\bar{x}$ = 0.680	= 0.435 +/− 0.0
5	$\bar{x}$ = 1.184	= 0.939 +/− 0.0
6	$\bar{x}$ = 1.267	= 1.022 +/− 0 0

To Page No.

Witnessed & Understood by me, [signature] Date 7/26/99

Invented by [signature] Recorded by Date 1/15/99

[redacted]

[redacted]

pg 87

LE EPO IgG Fusions Project No. Book No. 97

TEST NO.	:	W/L MODE	: DUAL	DATE : 01/15/99
TEST NAME	: MTS19498	TEST FILTER	: 490 nm	TIME : 14:35:07
PLATE	: JC 11599C3	REF. FILTER	: 650 nm	OPERATOR :

Calculation mode : Endpoint plate C pCDNA 3.1

DATA MATRIX : OD

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.190	0.197	0.193	0.194	0.195	0.197	0.196	0.193	0.198	0.193	0.194	0.195
		No cells	0	1	2	3	4	5	6			
B	0.197	0.247	0.294	0.303	0.309	0.312	0.313	0.313	0.304	0.197	0.193	0.193
C	0.197	0.253	0.301	0.308	0.314	0.308	0.323	0.309	0.308	0.193	0.193	0.194
D	0.187	0.258	0.299	0.307	0.311	0.314	0.317	0.311	0.304	0.195	0.197	0.195
E	0.194	0.196	0.189	0.191	0.192	0.191	0.193	0.192	0.192	0.195	0.192	0.193
F	0.195	0.196	0.184	0.190	0.191	0.190	0.195	0.194	0.194	0.193	0.192	0.188
G	0.192	0.192	0.179	0.186	0.190	0.189	0.191	0.196	0.191	0.193	0.193	0.185
H	0.170	0.193	0.185	0.184	0.192	0.194	0.195	0.191	0.194	0.193	0.184	0.189

pCDNA 3.1

No cells $\bar{x}$ = 0.253

0 $\bar{x}$ = 0.298 - 0.253 = 0.045 +/- 0.004

1 $\bar{x}$ = 0.306 = 0.053 +/- 0.003

2 $\bar{x}$ = 0.311 = 0.058 +/- 0.003

3 $\bar{x}$ = 0.311 = 0.058 +/- 0.003

4 $\bar{x}$ = 0.318 = 0.065 +/- 0.005

5 $\bar{x}$ = 0.311 = 0.058 +/- 0.002

6 $\bar{x}$ = 0.305 = 0.052 +/- 0.002

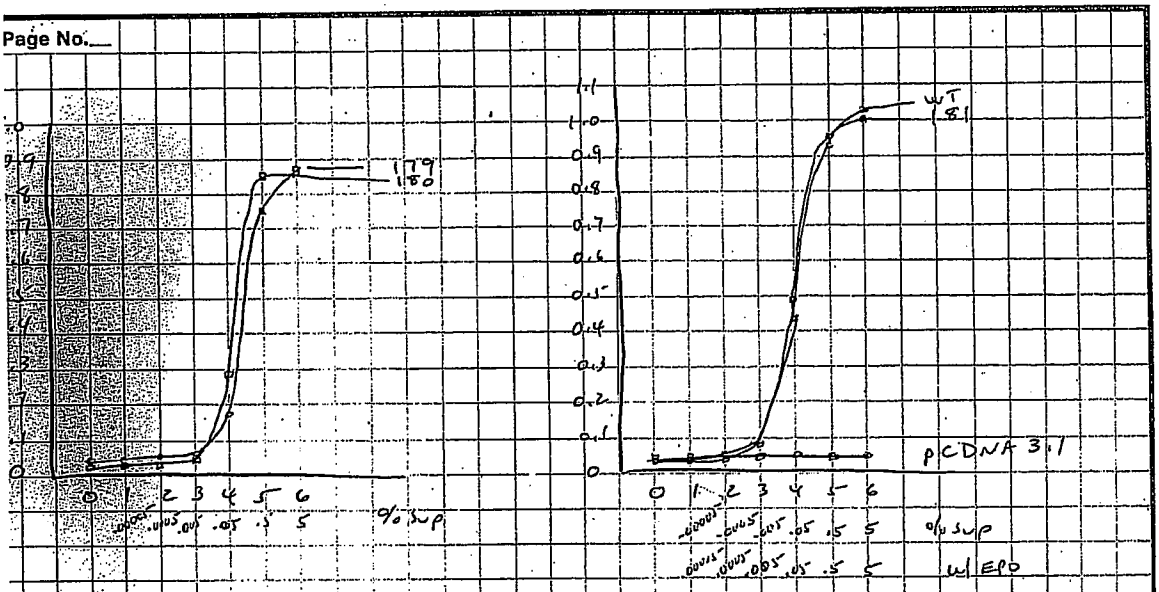
To Page No.

nessed & Understood by me, Date 2/26/99 Invented by Date 1/11/99 Recorded by

Project No._____ EPO-Fc pg 87

Book No._____ TITLE _____

Page No.___



Result:

Epo-Fc-6 is very active. If Dennis estimate of 1 ng/ml is correct, then EC_{50} is same as WT EPO

To Page No.___

Read & Understood by me,	Date	Invented by	Date
[signature]	2/26/99	[signature]	1/15/99
		Recorded by	

EXHIBIT I

172

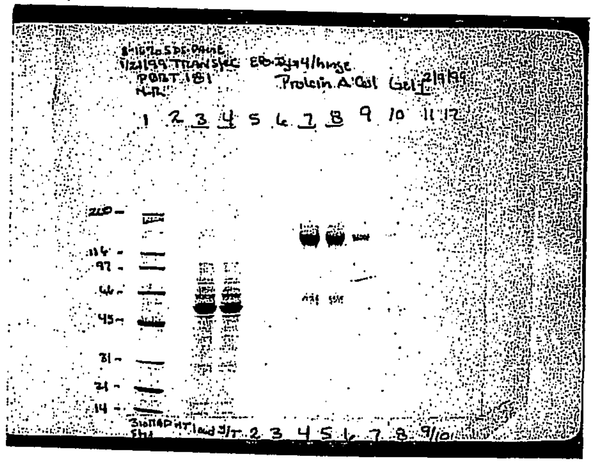
Project No.______
Book No.______ TITLE SDS PAGE of Protein A Col 1/21/99 Transfec

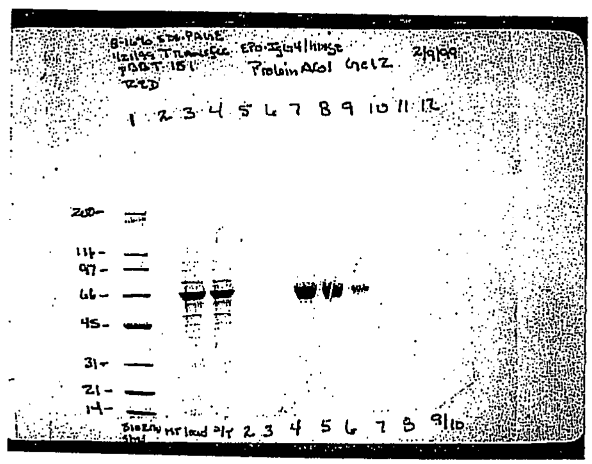
From Page No. 8-1690 SDS PAGE

2/9/99

Gel 1 (NONRED)

Lane Sample
1 BioRAD Prestained Std
2 MT
3 Load
4 F/T
5 Eln 2
6 3
7 4
8 5
9 6
10 7
11 8
12 9/10





Gel 2 (RED)

Lane Sample
1 BioRAD Std
2 MT
3 Load
4 F/T
5 Eln 2
6 3
7 4
8 5
9 6
10 7
11 8
12 9/10

Sample Prep:
Load/frac 20μl + 5μl 5xSBT-5%2ME load All
Eln 2 frac 10μl lode + 5μl 5xSBT-5%2ME "

Heat 100°C 5min

To Page No.

Witnessed & Understood by me, [signature] Date 8/17/99

Invented by [signature] Date 2/10/99

Recorded by

Project No.

TITLE 2/2/99 Transfer. Purification Book No.

From Page No. pBBT 179 (EPO-IgG1 (CH1)) 2/16/99

- Pool CM from Days 3, 6, 9 & 12
  Vol = 369 ml
  Conc. on YM 30 membrane @ Amicon Stirred Cell. 40C 55 PSI
  √ Vol =
  Wash ml 20mM NaPi pH 7.0
  Final vol = ml

2. Load onto 1ml HiTrap rProtein A Col.
   Dir = 0.6ml/min Collect FT as 1 frac.
   Wash @ 10ml (1ml/min) 20mM NaPi pH 7.0 Collect as 1 frac
   Elute @ 1ml/min @ 0.1 M NaCitrate pH 3.0. Collect 0.5ml frac. into 0.1ml 1M Tris pH 9.0 to Neutralize.
   √ pH of frac. & Adj. @ 1M Tris pH 9.0 as needed to get pH to ~7
   √ Done!

To Page No. 18

Witnessed & Understood by me,	Date	Invented by	Date
Mary S 2	8/17/99	Recorded by	2/16/99

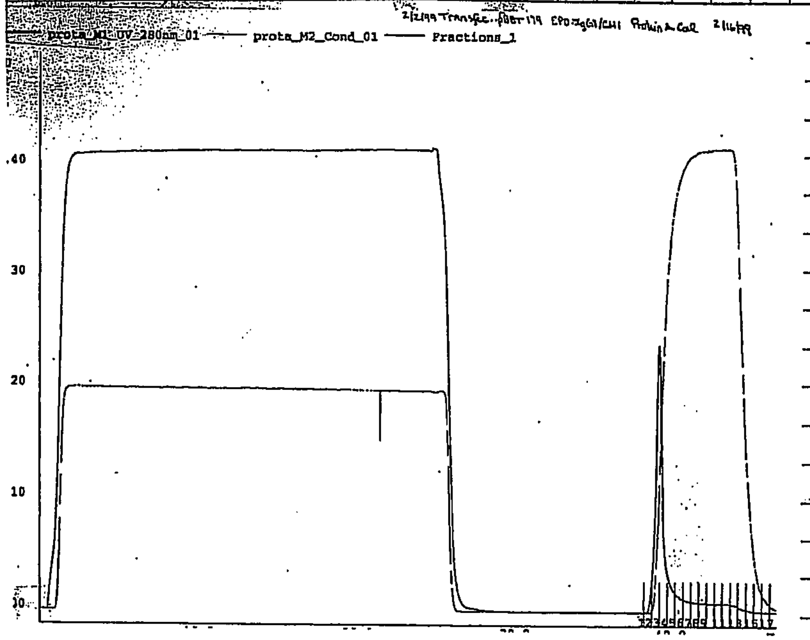
194

Project No.

Book No.

TITLE 2/2/99 Transfec Purific. ~~[illegible]~~ Protein A col

om Page No. 83



ser: DARIN 02-16-1999 04:08AM
ethod file: prota.frs

**** Run description *****

ample
2/2/99 Transfection pBBT 179 CM's 3,6,9&12

oop
50ml Superloop

olumn
1ml HiTrap rProtein A column

luent A
20mM NaPi pH 7.0

luent B
0.1M NaCitrate pH 3.0

radient

low rate
Load at 0.6ml/min Wash and elute at 1.0ml/min

raction size
Flo-thru as 1frac wash as 1frac elute as 0.5ml fractions

ther

ethod notes

un notes

valuation notes

ser: DARIN 02-16-1999 02:54AM
ethod file: affi.fmt

ethod base : ml

**** Main method *****

0.00	FLOW	0.6	{}
0.00	CONC_B	0	{}
0.00	INJ_VALVE	Load	{}
0.10	INJ_VALVE	Inject	{}
28.00	FLOW	1.00	{}
28.00	INJ_VALVE	Load	{}
38.00	CONC_B	0	{}
38.00	CONC_B	100	{}
43.00	FLOW	1	{}
43.00	CONC_B	100	{}
43.00	CONC_B	0.0	{}
48.00	FLOW	0.02	{}

To Page No.

essed & Understood by me,
[signature]

Date
8/17/[illegible]

Invented by
[signature]

Recorded by

Date
2/16/99

185

Project No.

TITLE SDS-PAGE Prot. A Col

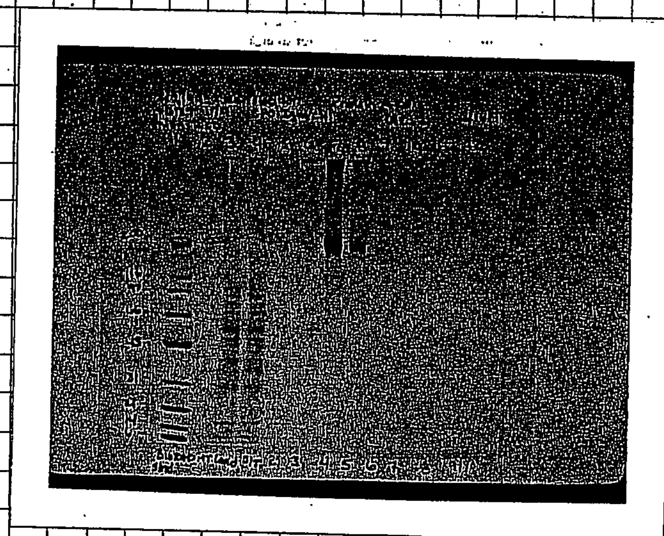
Book No.

From Page No. 8-16% SDS-PAGE

2/18/99

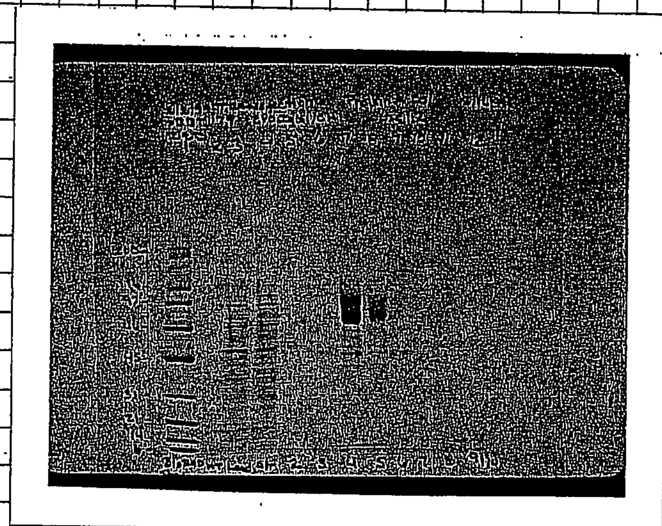
GEL 1 (Non RED)

Lane	Sample
1	BioRAD Prestained Std
2	MT
3	Protein A Col Load
4	S/T
5	S/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10



GEL 2 (RED)

Lane	Sample
1	BioRAD Std
2	MT
3	Col Load
4	S/T
5	S/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10



Sample Prep

Single Samples 20 µl Sample + 5 µl 5X SB + 5% 2ME Load All

2 frac pool 10 µl ea " + " " "

heat 100C 5 min

To Page No.

Witnessed & Understood by me, Mary S. [illegible]

Date 8/13/99

Invented by [signature]

Recorded by

Date 2/18/99

Project No.
Book No. TITLE 2/4/99 Transfec. Purification

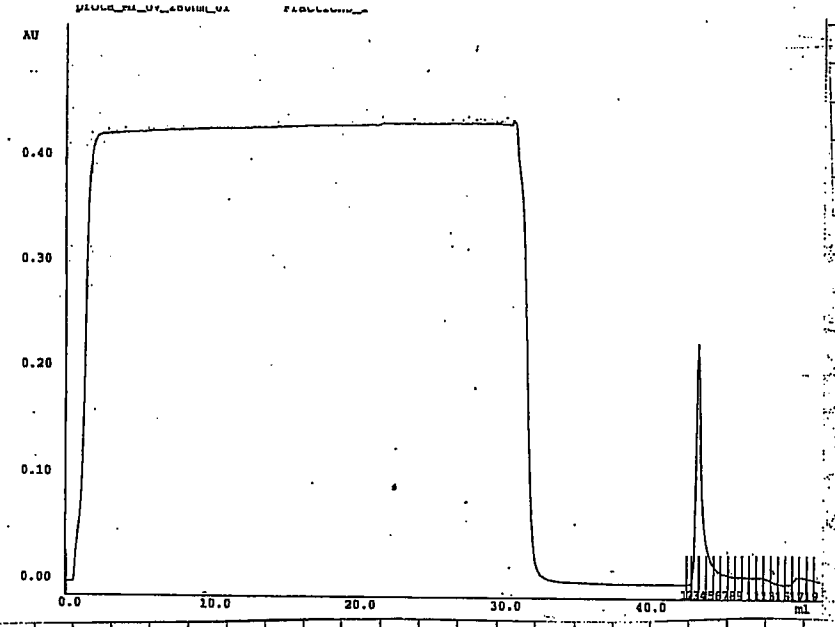
From Page No. pBRT 180 (EPO-IgG1/hinge) 2/17/99

Pool Cm's from Day 3, 6, 9 & 12
Vol = 270ml.
Conc on YM30 membrane Amicon Stirred Cell
to 27ml
Wash ē 5ml 20mM NaPi pH7.0. Final Vol = 32ml ~ 8.4x Conc

Load on 1ml HiTrap rProtein A Col. Equilib ē 100mM NaCitrate pH3.0 + 20mM NaPi pH7.0
and at 0.6ml/min Collect FT as 1 frac
Wash @ 1.0ml/min 10 Col Vol Collect as 1 frac
Elute ē 0.1M NaCitrate pH3.0. @ 1ml/min. Collect 0.5ml frac into 0.1ml 1M Tris pH9.0 to Neutralize
Check pH of frac & Adjust ē 1M Tris pH9.0 to ⇒ pH 7.0

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	2/17/99	[signature]	2/18/99
		Recorded by	



Method file: affi.fmt

Method base : ml

***** Main method *****

0.00	FLOW	0.6 []
0.00	CONC_B	0 []
0.00	INJ_VALVE	Load []
0.10	INJ_VALVE	Inject []
32.00	FLOW	1.00 []
32.00	INJ_VALVE	Load []
42.00	CONC_B	0 []
42.00	CONC_B	100 []
47.00	FLOW	1 []
47.00	CONC_B	100 []
47.00	CONC_B	0.0 []
52.00	FLOW	0.02 []

Method file: prota.rrs

***** Run description *****

Sample
2/4/99 Transfection pBBT 180 CM's 3,6,9,12 pool

Loop
50ml Superloop

Column
1ml HiTrap rProtein A column

Eluent A
20mM NaPi pH 7.0

Eluent B
0.1M NaCitrate pH 3.0

Gradient

Flow rate
Load at 0.6ml/min Wash and Elute at 1ml/min

Fraction size
Flow thru as 1frac wasg as 1frac Elution as 0.5ml fraction

Other

Method notes
Conc. CM pool from 2/4/99 Transfection run on 1ml Hitrap rProtein A column

Run notes

Evaluation notes

To Page No.

Witnessed & Understood by me, Mary S [illegible]

Date 8/17/99

Invented by [signature]

Date 2/18/99

Recorded by

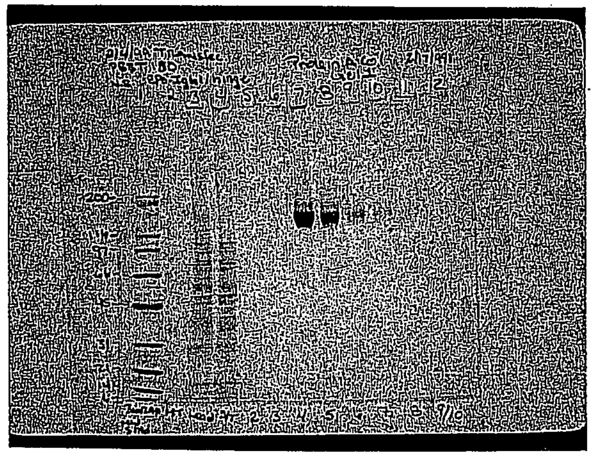
 11

Project No.
Book No.
TITLE SDS-PAGE 2/4/99 Transfec. Purif.

From Page No. 8-16% SDS-PAGE 2/17/99

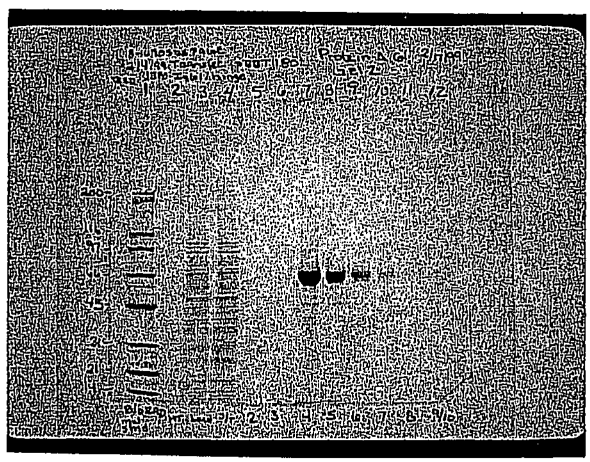
GEL 1 (NON RED)

Lane	Sample
1	BioRAD Stnd
2	MT
3	Protein A Col Load
4	FT
5	F/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10



GEL 2 (RED)

Lane	Sample
1	BioRAD Stnd
2	MT
3	Protein A Col Load
4	FT
5	F/N 2
6	3
7	4
8	5
9	6
10	7
11	8
12	9/10



Sample Prep:
All Single Samples 30μl + 5μl 5x SB +/- 5% 2ME Load All
Sample pool 10μl ea + 5μl 5x SB +/- 5% 2ME Load All

Heat 100°C 5min

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	8/17/95	[signature]	2/17/99
		Recorded by	

EXHIBIT J

From Page No. 95

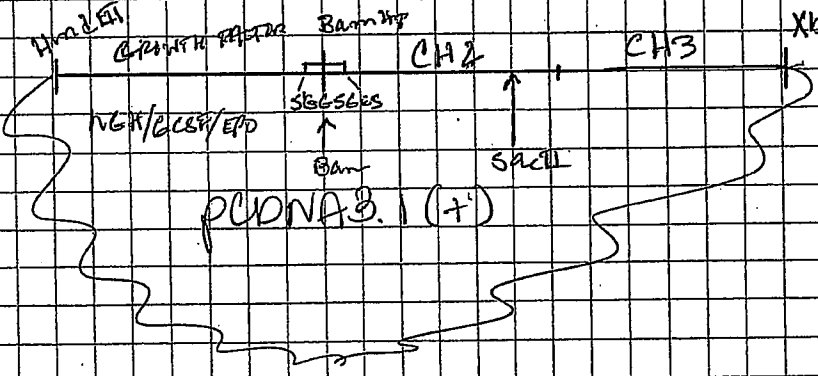
FEBRUARY 26, 1999

SEQ DATA SHOWS 99-3 OK BamHI → SacII

SO SUBCLONE THIS FRAG. INTO EXPRESSION VECTORS FOR GH-IgG4 hinge

GCSF - " "

EPO - " "



GH fus Ig G4 hinge = pBBST 163

GCSF " " " " = pBBST 175

EPO " " " " = pBBT 181

99-3 = QSP 4 (2/22/99) p94 = pBBST 182

Digest 163, 175, 181 (1-2ug) vs BamH + SacII + CIP + g-p VECTOR frag

" 182 vs BamHI + SacII + gp - 525bp frag

Clone 182 525 bp into 163, 175, 181 VECTORS

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
	3/5/99	Recorded by	3/3/99

EXHIBIT K

TITLE 3/23/99 Cos Cell Transfection

From Page No.___

pBBT 184 G-CSF-IgG4-CH 3/23/99
Setup Cos-1 Transfections pBBT 185 EPO-IgG4-CH

Use pBBT's from Endo prep 3/4/99

pBBT	Conc	for 700µl of 0.1µg/µl	TE
184	1.4µg/µl	50µl	650µl
185	1.5µg/µl	47µl	653µl

Setup L/D Mixes - for both pBBT 184 & 185

R	1 T-75	for 7 T-75's	
Plasmid DNA (0.1µg/µl)	93.5µl	655µl	
Lipofectamine	70.1µl	491µl	(Lot No. KH1Y07)
Opti MEM	2174µl	15.22ml	

Invert + Swirl to mix Let sit at R.T for 45 min

*

1. Rinse flasks 1x c̄ Opti-MEM 10ml/flask
   replace c̄ 7.7ml Opti-MEM / flask

2. Add 2.3ml L/D Mix / flask = 10ml final vol

   37°C 4 hr Start @ 10:15

3. After 4 hr aspirate off L/D Mix. Replace c̄ 10ml DMEM complete / flask

   => 37°C O/N

* Cells look to be ~45-50 % confluent. (Cells were plated on 3/22/99. 12 flasks @ 2x10⁶ cells/flask. Used cells from 3/18/99 P.43)

To Page No.___

Witnessed & Understood by me,	Date	Invented by	Date
Mary S R[signature]	9/2/99	[signature]	3/23/99
		Recorded by	

Project No.

E 3/23/99 Transfection Book No. 29

n Page No.

3/24/99

hange Media on 3/23/99 transfection.
1 Wash 2x ~ 10ml/wash DMEM Complete No Additives
replace ~ 10ml DMEM P/S/Glut / Flask.

Both sets of flasks look good. all are ~ 75-80% confluent. Some areas of flasks are more sparse (probably incubator level issue).

DAY=

hange Media on 3/23/99 transfect's (pBBT 184+185) 3/27/99
all flask look OK. ~ 90+% Confluent CM is yellow/orange
Lots of floating cells.
Ctf CM 5min 1200rpm - transfer to new falcon tubes. pull 500µl Sample
all => -20°C Vol ~ 70ml for each CM.

DAY=3

hange media on 3/23/99 Transfect's (pBBT 184+185) 3/30/99
pBBT 184 - flask #1 has mold contamination. Bleach & Discard. other flasks look
OK. a few only look to be ~ 80% confluent overall.
Ctf CM 5min 1200rpm - Transfer to new falcon tubes pull 500µl samples
all => -20°C Vol ~ 70ml for ~~each~~ pBBT ~~CM~~ 185 -20°C
~ 60ml for pBBT 184

DAY=6

hange Media on 3/23/99 Transfect. (pBBT 184+185) 4/2/99
pBBT 184 - flask #2 now has mold contam. bleach + discard. other
flasks all still OK.

DAY=9

4/5/99
Harvest DAY 12 CM.
pBBT 184 - cells look ok. Floaters & rounded cells present CM is yellow
no contamination.
185 - cells look OK same as pBBT 184

Ctf 5min 1000rpm. pull Supt 500µl => -80°C
remain. => -20°C

Discard cells

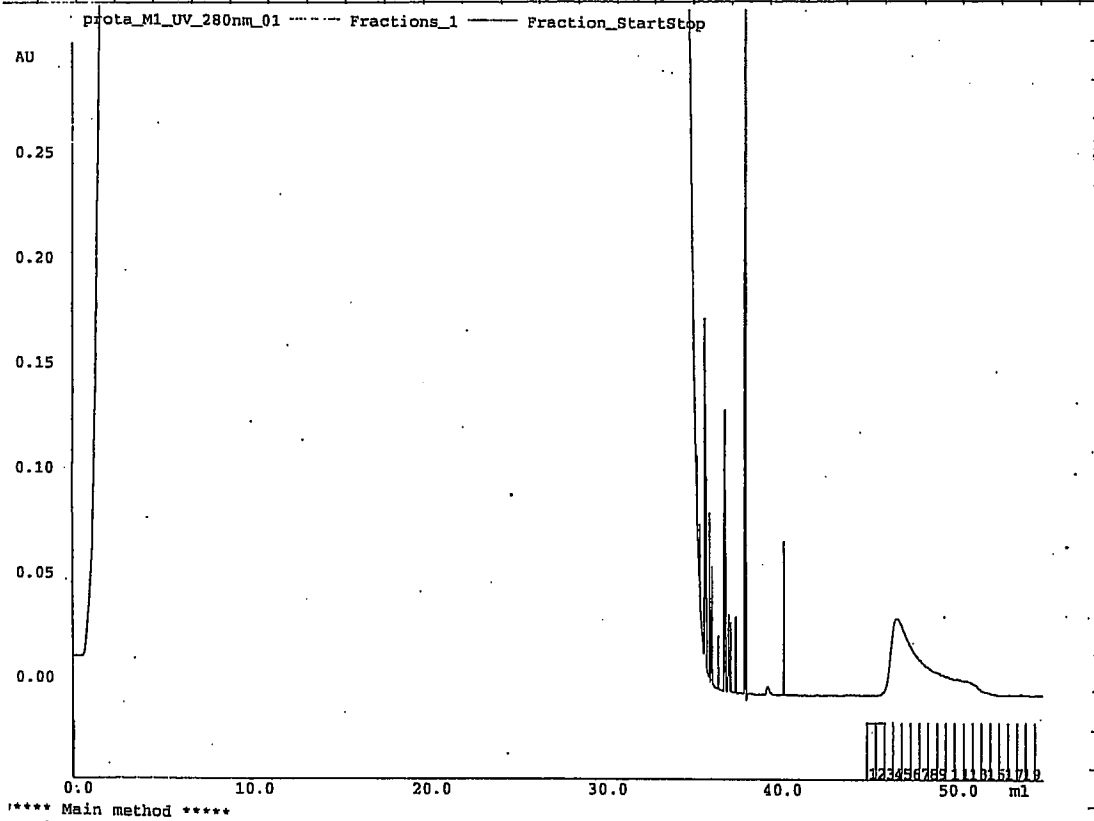
To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Mary R	9/6/99	Recorded by	4/5/99

m Page No. Purify EPO-Ig G4-CH (pBBT 185) 4/22/99

Thaw CH from 3/23/99 transfec Days 3, 6, 9 & 12
Vol = 270 ml
Conc on YM 3 membrane on Amicon Stirred cell
to 31 ml wash & bulk filtrate
Final vol = 37 ml = 7.3x conc

Run on 1ml rProtein A col HiTrap. Equilib = 1. 20mM NaPi pH 7.0
2. 20mM NaAcetate pH 3.0
3. 20mM NaPi pH 7.0.



Wash C
20mM NaPi

Elute 0.1M NaAcetate pH 3.0

***** Main method *****

0.00	FLOW	0.6
0.00	CONC_B	0
0.00	INJ_VALVE	Load
0.10	INJ_VALVE	Inject
35.00	FLOW	1.00
35.00	INJ_VALVE	Load
45.00	CONC_B	0
45.00	CONC_B	100
45.00	FLOW	1
50.00	CONC_B	100
50.00	CONC_B	0.0
55.00	FLOW	0.02

Load at 0.6ml/min
Wash & elute at 1ml/min
UV AC's: Flow Thru - 1 frac
Wash 1 frac
Elution 0.5ml frac

Collect Elution frac into 100µl 1M Tris pH 9.0

To Page No. 62

nessed & Understood by me,

Date 9/8/99

Invented by

Recorded by

Date 4/29/99

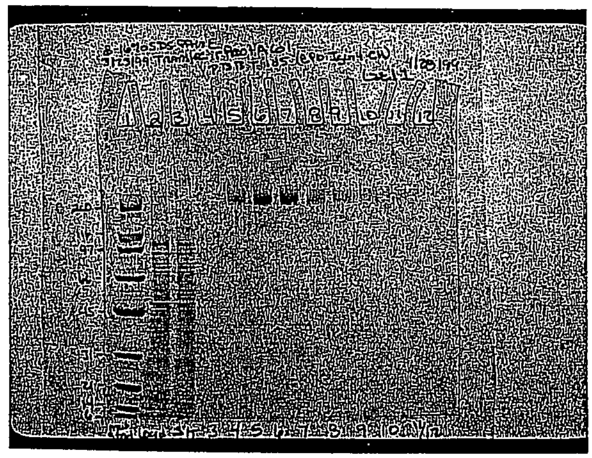
Project No.________
Book No.________ TITLE 3/28/99 Transfc Purf. Gels

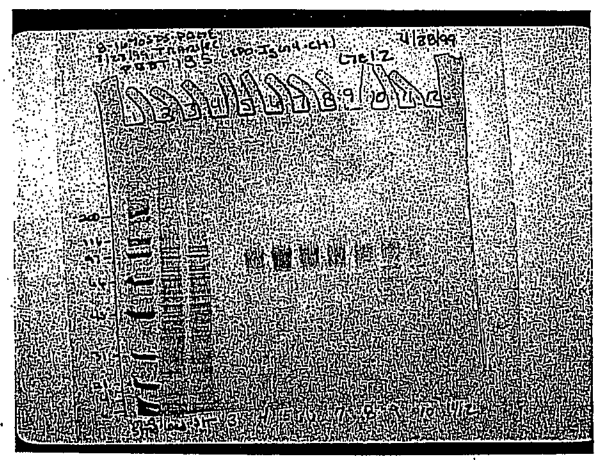
From Page No. 61 4/28/99

Adjusted pH of frac to ~7.5 c̄ either 1.0M Tris pH 9.0 or 0.1M Na Acetate pH 4.0

Run Samples on 8-16% SDS-PAGE Gel 1 NR Gel 2 Red

Lane	Sample
1	mw std
2	Col load
3	3/1T
4	3/1A 3
5	4
6	5
7	6
8	7
9	8
10	9
11	10
12	11/12





Sample Prep:
Load, 3/1T + Single frac 20µl + 5µl 5xSB/4-5% 2ME
2 frac pool 10µl ea + 5µl 5xSB/4-5% 2ME

heat All at 100°C 5min

To Page No.___

Witnessed & Understood by me, Mary S [signature] Date 9/2/99
Invented by [signature] Date 4/30/99
Recorded by

EXHIBIT L

Expiration Date 8/31/200

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase II Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1a. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Growth Factor Conjugates For Treating Hormonal Diseases

1b. Phase I Grant No.
1R43 DK54561-C

2. PRINCIPAL INVESTIGATOR ☐ New Investigator

2a. NAME *(Last, first, middle)*
Cox, George Norbert

2b. DEGREE(S)
B.A. | Ph.D. |

2c. SOCIAL SECURITY NO.
Provide on Personal Data Page

2d. POSITION TITLE
Chief Scientific Officer

2e. MAILING ADDRESS *(Street, city, state, zip code)*
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

2f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 303-735-2296
FAX: 303-492-8731

BITNET/INTERNET Address:
BolderBio@aol.com

3. HUMAN SUBJECTS	3a. If "yes," Exemption no. or IRB approval date	Full IRB or Expedited Review	3b. Assurance of compliance no.	4. VERTEBRATE ANIMALS	4a. If "Yes," IACUC approval date	4b. Animal welfare assurance no.
☒ NO ☐ YES		☐ ☐		☐ NO ☒ YES	pending	none

8. PERFORMANCE SITES *(Organizations and addresses)*
Bolder Biotechnology, Inc.
Porter Biosciences Building, Suite A140
University of Colorado
Boulder, CO 80309

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

10. ENTITY IDENTIFICATION NUMBER
84-1372278

Congressional District
Colorado, 2nd

11. INVENTIONS AND PATENTS ☐ Previously reported *OR* ☐ Not previously reported
☒ NO ☐ YES If "Yes,"

12. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned ☐ Socially and Economically Disadvantaged

13. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages 5-7, 14-17, 20-47 of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

14. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☒ YES ☐ NO

15. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: George N. Cox
Title: President & CSO
Address:
Bolder Biotechnology, Inc.
678 West Willow Street
Louisville, CO 80027

Telephone: 303-735-2296
FAX: 303-492-8731
BITNET/INTERNET Address:
BolderBio@aol.com

16. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 2a *(In ink. "Per" signature not acceptable.)*
George N. Cox

DATE
4/14/99

17. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 15 *(In ink. "Per" signature not acceptable.)*
George N. Cox

DATE
4/14/99

***Research Plan:**

***A. Specific aims:**

* There is considerable interest on the part of patients and healthcare providers in the development of low cost, *long-acting, "user-friendly" protein therapeutics. Most protein pharmaceuticals have short circulating half-lives in *the body and must be injected daily or every other day for maximum effectiveness. For example, Growth *Hormone (GH) and granulocyte colony-stimulating factor (G-CSF) require daily injections and erythropoietin * (EPO) requires every other day injections for maximum effectiveness. These recombinant proteins have proven *extremely effective at treating short stature and cachexia (GH), neutropenia (G-CSF) and anemia (EPO). For each *of these proteins it is known that increasing the circulating half-life of the protein improves the protein's *in vivo* *performance. We propose to create longer-acting forms of GH, G-CSF and EPO through covalent fusion of these *proteins to the heavy chain domain of human IgG1. Human IgG1 has a long serum half-life, on the order of 21 *days. Fusion of several other proteins, principally extracellular domains of cell surface receptors, to the IgG1 heavy *chain domain has resulted in increased serum half-lives for these proteins. Despite this success, IgG fusion protein *technology has been applied in only a few instances to cytokines and growth factors. Fully active IL-2- and IL-10- *IgG fusion proteins have been constructed, but data are not available for other cytokines and growth factors.

* During Phase I, we created recombinant fusion proteins comprising GH, G-CSF and EPO fused to the Fc * (Hinge-CH2-CH3) and C_H (complete heavy chain: CH1-Hinge- CH2- CH3) domains of human IgG1 and IgG4. *The fusion proteins were expressed as secreted as dimeric proteins from transiently transfected mammalian cells. *The fusion proteins were purified and their bioactivities compared to the corresponding non-fusion proteins in *appropriate *in vitro* mammalian cell proliferation assays. On a molar basis, the EPO-IgG-Fc and G-CSF-IgG-Fc *fusion proteins had biological activities essentially identical to EPO and G-CSF in the *in vitro* bioassays. In *contrast, bioactivities of the GH-IgG-Fc fusion proteins were reduced 6-10-fold compared to GH. Biological *activities of all of the IgG-C_H fusion proteins were reduced approximately 3-fold relative to the activities of the *corresponding IgG-Fc fusion proteins. The lower activities of the IgG-C_H fusions appeared to be due to *aggregation of the proteins during purification. The Phase I studies have identified the EPO-IgG-Fc and G-CSF- *IgG-Fc fusion proteins as excellent candidates for further development. During Phase II we will concentrate efforts *on these proteins and do not propose further studies of the GH-IgG fusion proteins at this time.

During the Phase II portion of the grant, initial work will focus on characterizing the *in vivo* properties of the *EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins. We will immediately manufacture sufficient quantities of these *fusion proteins for *in vivo* studies. We will measure the pharmacokinetic properties of the fusion proteins and *compare their relative effectiveness to wild type EPO and G-CSF in normal and diseased animals. The animal *studies will determine the effects of different dosing regimens on the *in vivo* effectiveness of the proteins. Our goal *is to create fusion proteins that are equal or superior to the natural proteins in stimulating biological activities *in* **vivo*, but which require less frequent dosing, on the order of once every two to four weeks, rather than daily or *every other day. Previous studies suggest it should be possible to achieve this goal by fusion of the proteins to the *heavy chain domain of human IgG1.

* A second aspect of the Phase II studies will be to optimize specific features of the fully active EPO-IgG-Fc and *G-CSF-IgG-Fc fusion proteins. In particular we will eliminate or minimize the linker between the growth factor *and the IgG domain. If the animal studies indicate that complement and Fc receptor binding properties of the IgG *domain causes toxicities *in vivo* we will introduce mutations that eliminate or reduce these functions.

* A third aspect of the Phase II studies will be to develop high level expression systems for manufacture of the *fusion proteins using stably transformed mammalian cells. We will optimize procedures for purifying the proteins *and develop analytical methods for characterizing their purity and structural properties.

* A fourth aspect of the Phase II portion of the grant will be to continue efforts to construct fully active EPO- *IgG1-C_H and G-CSF-IgG-C_H fusion proteins, since these proteins should have even longer circulating half-lives *than the corresponding IgG-Fc fusions. We believe we understand why the IgG1-C_H fusion proteins are *aggregating and propose experiments to rectify the problem and create fully active versions of these proteins. If *fully active EPO- and G-CSF-IgG1-C_H fusion proteins are constructed, we will characterize their *in vivo* properties.

* The primary goal of the Phase II portion of the grant is to identify the best EPO-IgG and G-CSF fusion proteins *for commercial development. The specific tasks proposed for Phase II are:

* 1. Prepare several hundred micrograms of each of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins *for pharmacokinetic and animal efficacy studies.

* 2. Prepare several hundred micrograms each of the control, non-fused EPO and G-CSFproteins for *pharmacokinetic and animal efficacy studies.

* 3. Perform pharmacokinetic experiments to demonstrate increased circulating half-lives of the fusion *proteins relative to the non-fused proteins.

* 4. Compare the relative effectiveness of EPO-IgG-Fc and EPO in stimulating red blood cell formation in *normal and anemic animals.
* 5. Compare the relative effectiveness of G-CSF-IgG-Fc and G-CSF in stimulating neutrophil formation in *normal and neutropenic animals.
* 6. Create stably-transfected mammalian cell lines expressing the most effective EPO-, G-CSF- and GH-IgG *fusion proteins.
* 7. Determine whether it is possible to eliminate or minimize the linker sequence between the growth factor *and the IgG domain without reducing biological activities of the fusion proteins.
* 8. Continue efforts to create fully active EPO- and G-CSF- IgG1-C_H fusion proteins.
* 9. Perform pharmacokinetic and animal efficacy experiments with any new fully active EPO- or G-CSF- *IgG-C_H fusion proteins (i.e. repeat tasks 1-5 above).

***B. Significance**

* There is considerable interest on the part of patients and healthcare providers in the development of low cost, *long-acting, "user-friendly" protein therapeutics. Proteins are expensive to manufacture and unlike conventional *small molecule drugs, are not readily absorbed by the body. Therefore, proteins must be administered by injection. *Most proteins are cleared rapidly from the body, necessitating frequent, often daily, injections for optimum *effectiveness. This is the case for GH and G-CSF. Some proteins including EPO are effective when administered *less often (three times per week for EPO) but this is due to the fact that the proteins are glycosylated. Patients *dislike injections, which leads to reduced compliance and reduced drug efficacy. The length of time an injected *protein remains in the body is determined by the protein's size and whether or not the protein contains covalent *modifications such as glycosylation. Circulating concentrations of injected proteins change constantly, often by *several orders of magnitude, over a 24 hour period. Rapidly changing concentrations of protein agonists can have *dramatic downstream consequences, at times understimulating and at other times overstimulating target cells. *Similar problems plague protein antagonists. These fluctuations can lead to decreased efficacy and increased *frequency of adverse side-effects for protein therapeutics. The rapid clearance of recombinant proteins from the *body significantly increases the amount of protein required per patient and dramatically increases the cost of *treatment. The cost of human protein pharmaceuticals is expected to increase dramatically in the years ahead as *new and existing drugs are approved for more disease indications. Current word-wide sales of protein therapeutics *are in excess of $10 billion annually and are growing at a greater than 10% annual rate. Thus, there is a strong *need to develop protein delivery technologies that lower the costs of protein therapeutics to patients and healthcare *providers. One solution to this problem is the development of methods to prolong the circulating half-lives of *protein therapeutics in the body so that the proteins do not have to be injected frequently. This solution also *provides patients and healthcare providers with protein therapeutics that are " user-friendly", i.e., that do not *require frequent injections.

* **EPO** is a 35-39 kDa glycoprotein secreted by the adult kidney. The mature human protein contains 165 amino *acids and is heavily glycosylated. EPO is the hormone primarily responsible for stimulating erythropoiesis or red *blood cell formation. EPO acts on immature red blood cell precursors to stimulate their further proliferation and *differentiation into mature red blood cells. Recombinant human EPO is used to restore red blood cell production in *patients with anemia resulting from renal failure, chemotherapy and drug complications. EPO recently received *FDA approval for stimulating red blood cell formation in patients undergoing certain types of elective surgeries.

*U.S. sales of EPO exceeded $2 billion and world-wide sales exceeded $3 billion in 1997. The protein is *administered by thrice weekly intravenous (dialysis patients) or subcutaneous (non-dialysis patients) injections.

* The structure of porcine GH has been solved by X-ray crystallography (Abdel-Meguid et al., 1987). The *protein has a compact globular structure, comprising four amphipathic alpha helical bundles joined by loops and *human GH has a similar structure (de Vos et al., 1992). The three dimensional structure of G-CSF has been *determined by X-ray crystallography (Hill et al., 1993; Lovejoy et al., 1993) and resembles that of GH, comprising *four amphipathic alpha helical bundles joined by loops. The extensive glycosylation of EPO has precluded *cyrstallographic studies but Cheetham et al. (1998) recently reported NMR based structural studies and described a *four helix bundle structure very similar to those of GH and G-CSF.

* GH has two receptor binding sites and binds two receptor molecules (Cunningham et al., 1991; de Vos et al., *1992). Dimerization of GH receptors is required for activation of the intracellular signaling pathways that lead to *cellular responses to GH. It is believed that G-CSF (Fukunaga et al., 1991) and EPO (Matthew's et al., 1996) *dimerize their receptors in a manner similar to the way GH dimerizes its receptor.

* Boissel et al (1993) showed that fusion of a six amino acid poly-histidine tag to the C-terminus of EPO did not *interfere with EPO bioactivity which suggested that other C-terminal fusions, such as those proposed in this grant *application, would be active. The principal investigator is unaware of data addressing the question of whether C-*terminal fusions of GH or G-CSF are active.

*** Phase I: Determine whether IgG fusion proteins of GH, EPO and G-CSF are biologically active**

* We proposed to create IgG fusion proteins of GH, EPO and G-CSF with extended half-lives, increased efficacy *and improved safety. To achieve this goal we needed to determine whether IgG fusions of these proteins were *biologically active and if so, how their bioactivities compared to bioactivities of the non-fused proteins. Previous *studies suggested that the C-terminus was not important for EPO bioactivity, but data for GH and G-CSF were not *available. During Phase I we constructed fusions of GH, EPO and G-CSF with the C_H and Fc domains of IgG1. *We also constructed fusions of these growth factors with the corresponding domains of human IgG4. The goal of *the Phase I studies was to identify at least one IgG fusion protein of GH, EPO or G-CSF that retained complete or *near complete (within two-fold of wild type) *in vitro* biological activity.

***Relationship of Phase I to Phase II Studies**

* As described below, the Phase I studies have allowed us to determine that certain IgG fusion proteins of EPO *and G-CSF retain complete *in vitro* bioactivity. These results have demonstrated the feasibility of creating growth *factor-IgG fusion proteins that retain high activity. In contrast, the GH-IgG fusion proteins displayed significantly *reduced *in vitro* biological activity compared to GH. Therefore, during Phase II we will focus efforts on the fully *active EPO- and G-CSF- IgG fusion proteins and not pursue additional experiments with the GH-IgG fusion *protein. The Phase II studies are designed to demonstrate the superior performance of the EPO-IgG and G-CSF-*IgG fusion proteins relative to the non-fused proteins in animal disease models. Positive data from the Phase II *animal experiments will provide the proof-of-concept needed for us to negotiate a partnering agreement with a *large pharmaceutical/biotechnology company to rapidly bring long-acting, highly potent EPO-IgG and G-CSF-IgG *fusion proteins to market.

3. Phase I Final Report

*Project funding period: October 1, 1998 – May 31, 1999

*Personnel: The personnel who contributed to this work are listed below.

Person	Role	Hours Devoted to Project[a]
George Cox, Ph.D.	Principal Investigator	578 hr
Daniel Doherty, Ph.D.	Scientist	403 hr
Darin Smith	Research Associate	570 hr

[a] Through March 31, 1999

*Summary of Accomplishments

* The goal of the Phase I portion of the grant was to identify one or more fully active IgG fusions (Fc or C_H *fusion) of GH, EPO or G-CSF. The specific tasks involved were:

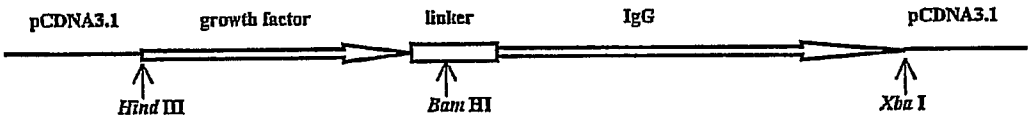
*1. Clone cDNAs encoding GH, G-CSF and EPO and fuse DNAs encoding these proteins to DNAs encoding the Fc
* or complete heavy chain (C_H) region of human IgG1.
*2. Clone DNAs encoding the fusion proteins into a mammalian cell expression vector.
*3. Transiently transfect mammalian cells with DNAs encoding the fusion proteins and purify the secreted fusion
* proteins to homogeneity using Protein A affinity chromatography, followed by other chromatographic
* procedures, if needed.
*4. Characterize the fusion proteins by polyacrylamide gel electrophoresis under reducing and non-reducing
* conditions to determine whether the fusion proteins are dimeric.
*5. Measure bioactivities (EC_{50}s) of the fusion proteins using appropriate GH-, G-CSF-, and EPO- responsive
* mammalian cell lines in culture. Compare bioactivities of the fusion proteins and non-fused control
* proteins in the bioassays.

* All of these objectives have been met and the primary goal has been exceeded. The six IgG1- Fc and IgG1-C_H *fusions have been constructed, expressed in mammalian cells and the recombinant fusion proteins purified and *analyzed. In addition, we also constructed fusions of GH, EPO and G-CSF to the Fc and C_H domains of IgG4, *which has certain properties, e.g., reduced affinity for complement and Fc receptors relative to IgG1, which may be *desirable for fusion protein therapeutics. The EPO and G-CSF IgG1-Fc and IgG4-Fc fusion proteins were found to *be as active as EPO and G-CSF in appropriate mammalian cell bioassays. These fusion proteins are excellent *candidates for further development. The EPO- and G-CSF-IgG1-C_H fusion proteins were found to have reduced *biological activities (2- to3-fold) relative to non-fused EPO and G-CSF. Reduced bioactivities of these proteins *appeared to be due to aggregation of the fusion proteins during purification. All of the GH-IgG fusion proteins had *4 -17-fold reduced biological activities relative to unfused GH, suggesting that the IgG domain may interfere with *the binding of GH to its receptor. A detailed summary of these experiments is provided below.

*I. Construction of GF-IgG gene fusions.

*I. A. Strategy. Twelve growth factor (GF)–IgG gene fusions were constructed. The general strategy employed for *these constructions is outlined here and the specifics of individual cloning steps are detailed below. Cloning of the *IgG4-C_H coding sequence involved additional variations to the general strategy and these variations are described *below. The human growth factor genes (GH, EPO and GCSF) were cloned as cDNAs from various RNA sources *detailed below. PCR primers used in these clonings added an optimized Kozak sequence (GCCACCC) and a *Hind* *III restriction site to the 5' end of each these clones and a portion of a flexible peptide linker (ser-gly-gly-ser) *terminating in a *Bam* HI restriction site, to the 3' end of each of these clones. The growth factor genes were cloned *as *Hind* III – *Bam* HI fragments into the mammalian cell expression vector pCDNA3.1(+) (Invitrogen, Inc.) and *sequenced. In parallel, IgG coding sequences (IgG1-Fc, IgG1-C_H, IgG4-Fc, IgG4-C_H) were cloned from cDNAs *generated from human leukocyte RNA. PCR forward primers used in these clonings incorporated a portion of a *flexible peptide linker (gly-ser-gly-gly-ser) containing a *Bam* HI restriction site at the 5' end of each of these *clones. The reverse PCR primers were designed to anneal to the 3' untranslated regions of the IgG1 and IgG4 *mRNAs (about 40 bp downstream of the translational stop codon) and included an *Xba* I restriction site. The IgG *coding sequences were cloned into pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and confirmed by DNA *sequencing. The fusion genes were then constructed by excising the IgG coding sequences as *Bam* HI – *Xba* I *fragments and cloning these fragments into the pCDNA::GF recombinant plasmids that had been cut with *Bam* HI *and *Xba* I. In the resulting pCDNA3.1 constructs the fusion genes are transcribed by the strong cytomeglovirus

*immediate early promoter present in pCDNA3.1(+) upstream of the cloned fusion gene. Ligation of the two
*fragments through the *Bam* HI site within the linker sequence results in a seven amino acid linker (ser-gly-gly-ser-
*gly-gly-ser) at the fusion junction.



***I. B. Cloning of growth factor cDNAs.**

* **Cloning of hGH:** A cDNA encoding hGH was amplified from human pituitary single-stranded cDNA *(CLONTECH, Inc.), using the polymerase chain reaction (PCR) technique and primers BB87 (5> *CGCAAGCTTGCCACCATGGCTACAGGCTCCCGGACG >3) and BB88 (5> CGCGGATCCTCCGGAGAA *GCCACAGCTGCCCTCCAC >3). Primer BB87 anneals to the 5' end of the coding sequence for the hGH *secretion signal, whereas the reverse primer, BB88, anneals to the 3' end of the GH coding sequence. The *resulting ~ 680 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into *pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel *purified. A clone with the correct DNA sequence was designated pCDNA3.1(+)::GHfus or pBBT159.

* **Cloning of EPO.** We previously cloned in pUC19 a cDNA encoding human EPO from the human liver *cell line Hep3B. The DNA sequence of the EPO gene was confirmed and the plasmid designated pBBT131. This *plasmid was used as template in a PCR reaction with primers BB89 (5>CGCAAGCTTGCCACCATGGGGGTGC *ACGAATGTCCT >3) and BB90 (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTGCAGGC >3) to construct *a modified EPO cDNA suitable for fusion with IgG genes. Primer BB89 anneals to the 5' end of the coding *sequence for the EPO secretion signal and the reverse primer, BB90, anneals to the 3' end of the EPO coding *sequence. The resulting ~ 610 bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned *into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel *purified. A clone with the correct DNA sequence was designated pCDNA3.1(+)::EPOfus or pBBT176.

* **Cloning of G-CSF.** A cDNA encoding G-CSF was amplified by PCR from total RNA isolated from the *human bladder carcinoma cell line 5637 (American Type Culture Collection). The cells were grown in Roswell *Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml *streptomycin. RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, *Inc. (Santa Clarita, CA) following the manufacturer's directions. First strand synthesis of single-stranded cDNA *was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp *and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand *synthesis as template were carried out with forward primer BB91 (5>CGCAAGCTTGCCACCATGGCTGGACC *TGCCACCCAG>3 and reverse primer BB92 (5>CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG *>3). Primer BB91 anneals to the 5' end of the coding sequence for the G-CSF secretion signal and the reverse *primer, BB92, anneals to the 3' end of the G-CSF coding sequence. The resulting ~ 640 bp PCR product was *digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence was *designated pCDNA3.1(+)::G-CSFfus or pBBT165.

***I. C. Cloning of IgG coding sequences.**

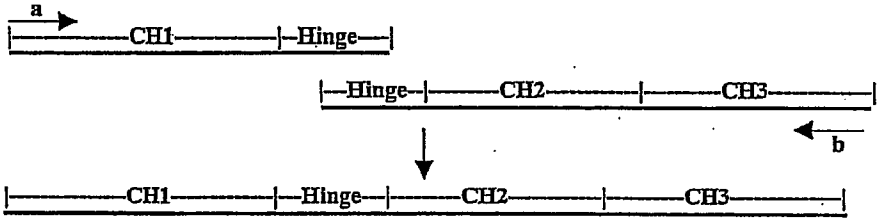
* **Cloning of IgG1-Fc coding sequences.** A cDNA encoding IgG1-Fc (hinge-CH2-CH3 domains) was *amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB83 *(5>CGCGGATCCG GTGGCTCAGAGCCCAAATCTTGTGACAAAACT >3) and BB82 (5>CGCTCTAG *AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB83 anneals to the 5' end of the coding sequence of *the hinge domain of IgG1, whereas the reverse primer BB82 anneals to the 3' untranslated region of IgG1 and *IgG4 mRNA ~ 45 bp downstream of the translational stop codon. The IgG1 and IgG4 sequences are identical over *the 21 bp segment to which BB82 anneals. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I, gel *purified and cloned into pCDNA3.1(+) vector that had been digested with *Bam* HI and *Xba* I, alkaline phosphtase *treated, and gel purified. Two clones were sequenced but each contained a single base pair substitution that *resulted in an amino acid substitution mutation. Otherwise the sequences matched the published human IgG1 *genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us *to use convenient unique restriction sites (*Sac* II in the CH2 domain of IgG1 and *Bgl* II in the pCDNA3.1(+)

*vector) to construct a full length IgG1-Fc clone in pCDNA3.1(+) via *in vitro* recombination. The resulting clone, *which had the correct IgG1-Fc sequence, was designated pCDNA3.1(+)::fusIgG1-Fc or pBBT167.

* **Cloning of IgG4-Fc coding sequences.** A cDNA encoding IgG4-Fc (hinge-CH2-CH3 domains) was *amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB84 *(5>CGCGGATCCGG TGGCTCAGAGTCCAAATATGGTCCCCCATGC >3) and BB82 (5>CGCTCTAG *AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB84 anneals to the 5' end of the coding sequence of *the hinge domain of IgG4. The reverse primer BB82 is described above. The ~ 790 bp PCR product was digested *with *Bam* HI and *Xba* I and cloned into pCDNA3.1(+) that had been similarly digested. A clone with the correct *DNA sequence (Ellison et al., 1981) was designated pCDNA3.1(+)::fusIgG4-Fc or pBBT158.

* **Cloning of IgG1-C_H coding sequences.** A cDNA encoding IgG1-C_H (CH1-hinge-CH2-CH3 domains) was *amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using BB81 (5>CGCGGATCC *GGTGGCTCAGCCTCCACCAAGGGCCCATC>3) and BB82 (5>CGCTCTAGAGGTACGTGCCAAGC *ATCCTCG>3). Forward primer BB81 anneals to the 5' end of the coding sequence of the CH1 domain of IgG1 *and IgG4. The sequences at the 5' end of the CH1 domains of these two exons are almost identical: 19/20 *nucleotides match. The reverse primer, BB82, is described above. The ~ 1080 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) that had been digested similarly. These primers in *principle could amplify both IgG1 and IgG4 sequences. Since IgG1 is much more abundant in serum than IgG4 *(**Paul, 19xx**) we expected that most clones would encode IgG1. The first two clones sequenced were IgG1 but *each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the *sequences obtained matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative *positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Age* I in the CH1 *domain of IgG1 and *Bst* BI in the pCDNA3.1(+) vector) to construct a full length IgG1-C_H clone in pCDNA3.1(+) *via *in vitro* recombination. A clone with the correct IgG1-C_H sequence was designated pCDNA3.1(+)::fusIgG1-C_H *or pBBT166.

* **Cloning of IgG4-C_H coding sequences.** The near identity of the DNA sequences encoding the 5' ends of *the IgG1 and IgG4 CH1 domains and the relatively low abundance of the IgG4 mRNA led us to an alternative *strategy for cloning the IgG4-C_H coding sequences. We used PCR-based site directed mutagenesis to change the *DNA sequence of the cloned IgG1 CH1 domain to match the amino acid sequence of the IgG4 CH1 domain. The *CH1 domains differ at only 8 of 98 nucleotides and these positions are clustered, so that one round of PCR using *two mutagenic oligos can convert the IgG1 CH1 sequence into the IgG4 CH1 sequence. A second round of PCR *added the *Bam* HI site and linker sequence to the 5' end of the IgG4 CH1 and 21 bp of sequence from the IgG4 *Hinge domain to the 3' end. The technique of "gene splicing by overlap extension" (Horton et al., 1993) was then *employed to recombine the engineered IgG4 CH1 domain with the IgG4 Fc (Hinge-CH2-CH3) sequence. In this *technique two separate fragments sharing a segment of identical sequence, the "overlap", at one end are extended *through the annealed overlap regions in a PCR reaction as diagrammed below.



*To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5> TCCACCAAG GGCCCATCCGT *CTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGC ACAGC>3) and BB120 (5> TCTCTTG *TCCACCTTGGTGTTGCTGGGCTTGTGATC TACGTTGCAGGTGTAGGTCTTCGTGCCCAA >3) were used *in PCR reactions with pBBT166, which carries the cloned IgG1-C_H sequence as described above. Forward primer *BB119 anneals to the sequence encoding amino acids 2 through 23 of the CH1domain and encodes 4 amino acid *substitutions: S14C, K16R, G20E and G21S. Reverse primer BB120 anneals to the sequence encoding amino *acids 76 through 97 of the CH1 domain and encodes 4 additional amino acid substitutions: Q79K, I82T, N86D and *K97R. The ~ 290 bp product of this PCR reaction was gel purified and used template in a PCR reaction with 8primers BB81(see above) and BB121(5>TGGGGGACCATATTTG GACTCAACTCTCTTGTCCACCTT >3). *Reverse primer BB121 anneals to the 3' end of the CH1 domain of IgG4, adds amino acid 98 of the CH1 domain *and 21 bp extending into the Hinge domain of IgG4. The ~ 330 bp product of this reaction was gel purified and *used as one of the template molecules in the PCR splicing reaction. The other template for the splicing reaction

*was generated by PCR of the cloned IgG4-Fc sequence of pBBT158 (described above) with primers BB84 and *BB82 which amplify the IgG4 Fc domain as described above. The resulting ~ 790 bp product consists of the IgG4 *hinge-CH2-CH3 sequence. This fragment was gel purified and used as one of the template molecules in the PCR *splicing reaction. This reaction employed the primers BB81 and BB82 and generated a full-length "spliced" *product of ~ 1075 bp. To minimize the DNA sequencing required to confirm this product, the PCR fragment was *digested with *Bam* HI and *Sac* II and the ~ 530 bp fragment (containing the complete CH1 and hinge domains and *a portion of the CH2 domain) was cloned into pBC-SK+ (Stratagene) for sequencing. The sequence of the *Bam* HI *– *Sac* II fragment was confirmed for one clone which was the designated pBBT182. The *Bam* HI – *Sac* II fragment *of pBBT182 was then used convert the GF–IgG4-Fc clones to full length GF–IgG4-C_H clones as detailed below.

***I. D. Construction of GF–IgG fusions.** Most (9/12) of the growth factor-IgG gene fusions were generated by *excising the IgG coding sequences cloned in pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and cloning these *fragments into the pCDNA::[GF] recombinant plasmids which had been cut with *Bam* HI and *Xba* I. The fusions *of the three growth factor genes to IgG4-C_H were constructed by excising the ~ 530 bp *Bam* HI– *Sac* II fragment of *pBBT182 and replacing the ~ 240 bp *Bam* HI- *Sac* II fragments of the three pCDNA::[GF]-IgG4-Fc clones. The *resulting plasmids and the GF-IgG fusion proteins they encode are listed in Table 1.

***II. Expression and Purification of GF-IgG Fusion Proteins**

***II. A. Small Scale Transfection of COS Cells**

* Expression and bioactivity of the GF-IgG fusion proteins were assessed initially by small-scale transfection of COS *cells. Endotoxin-free plasmid DNAs were prepared using a Qiagen "Endo-Free Plasmid Purification Kit" according to *the vendor protocol and used to transfect COS-1 cells (ATCC). The COS-1 cells were Delbecco's Modified Eagle's *Media supplemented with 10% FBS, 50units/ml penicillin, 50µg/ml streptomycin and 2mM glutamine (growth media). *Initial transfection experiments were carried out in Costar 6 well tissue culture plates using the following protocol. *Briefly, 2-3 x 10^5 cells were seeded into each well in 2 ml of growth media and allowed to incubate overnight at 37°C *and 5% CO_2 by which time the cells had reached 50-60% confluency. For each well, 0.8 µg of plasmid DNA was *complexed with 6 µl of LipofectAMINE reagent (Gibco BRL) in 186 µl of OPTI-MEM I Reduced Serum Medium *(Gibco BRL) for 30-45 minutes at room temperature. COS-1 cells were washed one time with 2ml of OPTI-MEM I per *well and then 1.8 ml of OPTI-MEM I was added to each well. The complex mixture was then added to the well and left *at 37°C, 5% CO_2 for approximately 4-5 hours. After the incubation period, the mixture was replaced with 2 ml of *growth media per well and left overnight at 37°C, 5% CO_2. The next day the cells were washed twice with 2ml of *DMEM (no additives) per well. Following the wash steps, 2 ml of serum-free growth media was added to each well *and the cells left at 37°C, 5% CO_2. Conditioned media containing the GF-IgG-fusion proteins were harvested after 72 *hours and analyzed by SDS-PAGE and Western blot to confirm expression of the GF-IgG-fusion proteins. The parent *plasmid, pCDNA 3.1(+) (Invitrogen) was used as a negative control. Transfection efficiency was estimated to be *~15%, using pCMVβ (Clontech), which expresses *E. coli* β-galactosidase. Transfected cells expressing *β-galactosidase were identified using a β-Gal Staining Set (Boehringer Mannheim).

* Samples of the conditioned media were prepared in SDS-PAGE sample buffer with the addition of 1% β- *mercaptoethanol (BME) when desirable and electrophoresed on precast 14% Tris-glycine polyacrylamide gels *(Novex). Western blots using appropriate antisera demonstrated expression of all of the GF-IgG fusion proteins *(data not shown – see purified proteins below). The GH-IgG fusion proteins were detected using a polyclonal *rabbit anti-synthetic-hGH antiserum (kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary *Program). The EPO- and G-CSF-IgG fusion proteins were detected using polyclonal antisera purchased from *R&D Systems. Serial dilutions of the conditioned media were analyzed in the appropriate *in vitro* bioassays *described later. These assays demonstrated significant activity in the conditioned media (data not shown) and *encouraged us to perform large-scale transfections so that the proteins could be purified for specific activity *measurements.

***II. B. Large Scale Transfection of COS-1 Cells**

* Large scale transfections were carried out using Corning 100 mm tissue culture dishes or Corning T-75 tissue *culture flasks. For 100 mm dishes, 1.6 x 10^6 cells were plated in 10 ml of growth media per dish and incubated at *37°C, 5% CO_2 overnight. For each 100 mm dish, 6.7 µg endotoxin-free plasmid DNA was complexed with 50 µl *of LipofecAMINE reagent in 1.5 ml of OPTI-MEM I for 30-45minutes at room temperature. The COS-1 cells *were washed one time with 10 ml OPTI-MEM per dish and then replaced with 6.6 ml of OPTI-MEM I. Following *complex formation, 1.67 ml of the complex was added to each dish and left at 37°C, 5% CO_2 for 4-5 hours. After

*the incubation period, the reaction mixture was replaced with 10 ml of serum containing growth media per dish and *left at 37°C, 5% CO_2 overnight. The next day the cells were washed twice with 10 ml of DMEM (no additives) per *dish. Following the wash steps, 10ml of serum-free growth media was added to each dish and incubated at 37°C, *5% CO_2. Conditioned media were harvested routinely every three days (on days 3, 6, 9 and 12) and fresh serum-*free growth media added to the cells. Transfections in T-75 culture flasks were identical to the 100mm dish *protocol with the following exceptions: Cells were plated at 2 x 10^6 cells per flask and 9.35 µg of endotoxin-free *plasmid DNA was complexed with 70 µl of LipofectAMINE reagent in 2.1ml of OPTI-MEM I for each T-75 flask. *Following complex formation, 2.3 ml of the complex was added to each flask containing 7.7 ml of OPTI-MEM I. *Transfection efficiency was determined to be ~15% using pCMVβ and staining for β-galactosidase expression as *described earlier. The 12 plasmids listed in Table 1 were transfected into COS-1 cells using the large-scale format *to generate protein for purification. The conditioned media were clarified by centrifugation and stored at -20°C for *later purification. Western blots were used to confirm expression of the IgG-fusion proteins.

***II. C. Purification of GF-IgG-Fusion Proteins**

* Approximately 300 ml of transfected COS-1 cell conditioned media for each IgG-fusion protein was pooled and *concentrated using an Ultrafiltration cell and either a YM3 or YM30 DIAFLO Ultrafiltration membrane (Amicon). *Concentrated pools were then loaded onto a 1ml Pharmacia HiTrap recombinant Protein A column previously *equilibrated with 20 mM NaPhosphate pH 7.0. The column was washed with 20 mM NaPhosphate until the A_{280} *had reached baseline. Bound protein was eluted with 100 mM NaCitrate pH 3.0 and collected into sufficient 1M *Tris pH 9.0 to achieve a final pH of approximately 7.0. Each fusion protein was purified using a dedicated column *to avoid any possibility of cross-contamination. All of the IgG fusion proteins chromatographed similarly, yielding *a single peak in the elution step. Column fractions were analyzed using 8-16% precast Tris-glycine SDS-PAGE *and fractions enriched for the IgG-fusion protein were pooled. A typical chromatogram and corresponding gel *from the EPO-IgG1-Fc purification is shown in Figures 1A and 1B. Protein concentrations of the pooled fractions *were determined by Bradford assay using bovine serum albumin (BSA) as the standard. Recoveries of the various *purified GF-IgG fusion proteins are given in Table 1 and ranged from 96 to 376 µg per 300 ml of conditioned *media.

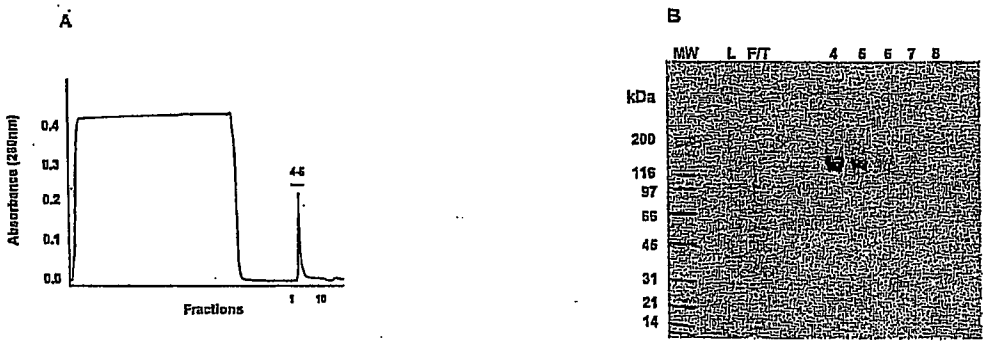


***Figure 1. rProtein A purification of EPO-IgG-Fc. Panel A shows the rProtein A column chromatogram. Panel B is non-reducing SDS-*PAGE analysis of the rProtein A column fractions. MW (molecular weight standards), L (column load), F/T(column flow through), and *column fractions 4-8.**

* To date, 9 of 12 GF-IgG fusion proteins have been purified to near homogeneity. Figure 2 displays SDS gels of *the purified proteins under reducing and non-reducing conditions and stained with Coomassie blue. All of the *GF-IgG fusion proteins were recovered principally as disulfide-linked dimers. The molecular weights of the *proteins ranged from 115-190 kDa kDa under non-reducing conditions and 50-70 kDa under reducing conditions, *largely consistent with the molecular weights predicted in Table 1. The molecular weights of the EPO-IgG fusion *proteins were the only ones larger than predicted (see Figure 2), presumably due to extensive glycosylation of the *EPO domain. Monomeric fusion proteins were more abundant with the IgG4-Fc fusion proteins (they can be seen *in the non-reduced gels in Figure 2), but still represented the minority (less than 10%) of the protein in these *preparations. The sizes of the major IgG fusion protein bands were different from the molecular weights of bovine

*IgG (see Figure 2), indicating that the proteins purified were not contaminating bovine IgGs from serum used in the *experiments. The major IgG fusion protein bands also reacted with antisera specific for GH, EPO and G-CSF in *Western blots of the samples (data not shown). Purity of the IgG fusion proteins was estimated to be at least 90% *from Coomassie blue staining of the gels.

* All of the GF-IgG-CH fusions contained a large aggregate that migrated at the top of the gel when the samples *were analyzed under non-reducing conditions. This aggregate disappeared when the samples were analyzed under *reducing conditions and the amount of protein at the molecular weight of the major GF-IgG-C_H bands seemed to *increase proportionately. The aggregates also reacted with antisera specific for the various growth factors. These *data suggest the aggregates are disulfide-linked multimers of the GF-IgG-C_H fusion proteins. Under reducing *SDS-PAGE conditions, all of the GF-IgG-C_H fusions show a diffuse band approximately 20 kDa larger than the *main GF-IgG-C_H band. This band reacted with antisera against the growth factors and may be related to the *aggregates.

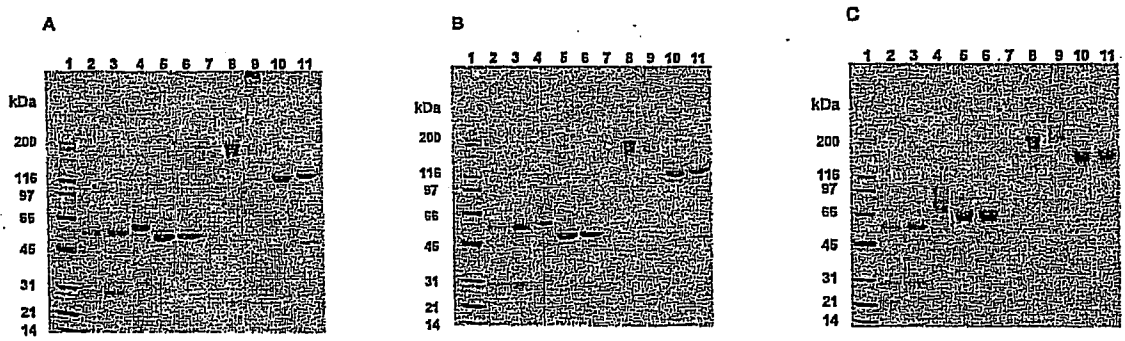


*Figure 2. Analysis of Purified IgG-fusion proteins by SDS-PAGE. Panel A shows purified GH-IgG fusion proteins. Lane 1, molecular *weight standards; Lanes 2 & 3 are bovine IgG standard at 1 & 2µg respectively, reduced; Lanes 4,5 & 6 are GH-IgG1-C_H, GH-IgG1-Fc, and *GH-IgG4-Fc respectively, reduced; Lane 8, 2µg bovine IgG standard non-reduced; Lanes 9,10 & 11, are identical to lanes 4,5 & 6 except non-*reduced. Panel B shows purified G-CSF-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are G-CSF-*IgG1-C_H, G-CSF-IgG1-Fc and G-CSF-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced. *Panel C shows purified EPO-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are EPO-IgG1-C_H, EPO-*IgG1-Fc and EPO-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced.

***Table 1 Predicted Molecular Weights and Recoveries of GF-IgG Fusion Proteins**

Expression Plasmid	GF-IgG Fusion Protein	Predicted Molecular Weight (kDa)* Monomer	Dimer	Protein Recovery (mg/ 300 ml)
pBBT 171	GH-IgG1-C_H	58,706	117,412	376
pBBT 172	GH-IgG1-Fc	48,693	97,386	248
pBBT183	GH-IgG4-C_H	58,541	117,082	In Progress
pBBT 163	GH-IgG4-Fc	48,365	96,730	96
pBBT 173	G-CSF-IgG1-C_H	55,564	111,128	122
pBBT 174	G-CSF-IgG1-Fc	45,551	91,102	122
pBBT 184	G-CSF-IgG4-C_H	55,399	110,798	In Progress
pBBT 175	G-CSF-IgG4-Fc	45,222	90,444	96
pBBT 179	EPO-IgG1-C_H	54,972	109,944	133
pBBT 180	EPO-IgG1-Fc	44,960	89,920	235
pBBT 185	EPO-IgG4-C_H	54,808	109,616	In Progress
pBBT 181	EPO-IgG4-Fc	44,632	89,264	257

*1 Does not include molecular weight contributions due to of glycosylation.

*III. Bioactivities of Purified IgG Fusion Proteins

* Cell proliferation assays were developed to measure bioactivities of the IgG fusion proteins. The assays measure *uptake and bioreduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-3-carboxyphenyl)-2-(4-*sulphenyl)-2H-tetrazolium]. In the presence of an electron coupler such as phenazine methosulfate (PMS), MTS is *converted to a formazan product that is soluble in tissue culture media and can be measured directly at 490 nm. *Cell number is linear with absorbance values up to about 2 (data not shown). The advantage of using MTS in the *assays, rather than the more conventional tetrazolium salt MTT (Mosmann, 1983) is that absorbance of the wells *can be determined without the need to lyse the cells with organic solvents, as is required for assays utilizing MTT. *For EPO and G-CSF we were able to use existing cell lines to develop the bioassays. For GH, we needed to create *a cell line that proliferates in response to GH. Such a cell line was created by stably transforming a murine *leukemia cell line with a GH receptor.

* In general, the bioassays were set up by washing the appropriate cells three times with media (no additives) and *resuspending the cells at a concentration of $1x10^5$/ml in media with additives (media used for each cell line is given *below). Fifty µl ($5x10^3$ cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue *culture plate. Serial dilutions of the protein samples to be tested were prepared in serum containing media. Fifty µl *of the diluted protein samples were added to the test wells and the plates incubated at 37^0C in a humidified 5% CO_2 *tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of an MTS/PMS *mixture (CellTiter 96 AQueous One Solution, Promega) was added to each well and the plates incubate at 37^0C in *the tissue culture incubator for 1-4 h. Absorbance of the wells was read at 490 nm using a microplate reader. *Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were *subtracted from mean values obtained for the test wells. EC_{50}s, the amount of protein required for half maximal *stimulation, was calculated for each sample and used to compare bioactivities of the proteins. Experiments were *repeated at least three times for each protein. Non-glycosylated molecular weights were used in the molar ratio *calculations for consistency; using molecular weights of the fusions estimated from SDS gels (50-70 kDa) and *35kDa for EPO gave similar activity ratios. Non-glycosylated molecular weights of 18,936, 18,987 and 22,129 *were assumed for EPO, G-CSF and GH, respectively. Monomer molecular weights were used in the calculations *for the IgG fusion proteins.

*III. A. EPO-IgG Fusion Proteins

* The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School, Boston, MA. This cell *line proliferates in response to EPO and is dependent upon EPO for cell survival (Boissel et al., 1993). The cells *were maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/ml *penicillin, 50 µg/ml streptomycin and 1 unit/ml recombinant human EPO (CHO cell-expressed; R&D Systems). *Bioassays were performed in cell maintenance media using the procedures described above. Serial dilutions of *recombinant CHO cell-expressed human rEPO (R&D Systems) were analyzed in parallel.

* The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase *in cell number and absorbance values (Figure 3). In the absence of rEPO, the majority of UT7/epo cells die, giving *absorbance values less than 0.1. Commercial CHO cell-expressed rEPO had a mean EC_{50} of approximately 0.6 *ng/ml in the bioassay (Table 2). This value agrees with EC_{50} values reported in the R&D Systems specifications *(0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml). The EPO-IgG1-Fc and IgG4-Fc fusion proteins had identical *EC50's of approximately 1.3 ng/ml in the bioassay (Table 2). On a molar basis, the EC_{50}s of CHO-cell expressed *rEPO and the EPO-IgG-Fc fusions were identical (approximately 30 pM; Table 2). The EPO-IgG1-C_H fusion *protein had an EC_{50} of 3.1 ng/ml or 60 pM (Table 2), which represents an approximate 2-fold reduction in specific *activity relative to the EPO-IgG-Fc fusion proteins and non-fused rEPO. Dose response curves for CHO cell-*expressed rEPO and the EPO-IgG fusion proteins performed on the same day are shown in Figure 3.

*Table 2. Bioactivities of EPO-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (ng/ml)[1]	Mean EC_{50}	
			ng/ml	pM
-	rhEPO (CHO)	0.52, 0.55, 0.60	0.56	30
pBBT180	EPO-IgG1-Fc	1.1, 1.2, 1.5	1.27	28
pBBT181	EPO-IgG4-Fc	1.1, 1.2, 1.5	1.27	29
pBBT179	EPO-IgG1-C_H	2.9, 3.0, 3.5	3.13	57
pBBT185	EPO-IgG4-C_H	In Progress	-	-

[1] Data from individual experiments

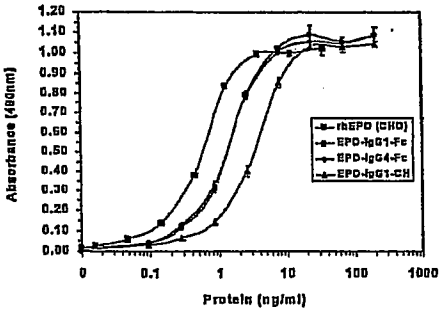


*Figure 3. Dose response curves for EPO-IgG fusion *proteins and rEPO for stimulating proliferation of *UT7/epo *cells. Data represent means +/- SD for *triplicate wells.

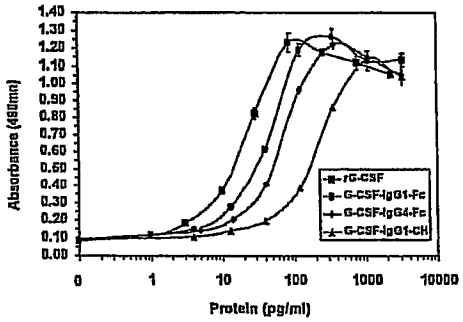


*Figure 4. Dose response curves for G-CSF-IgG *fusion proteins and rG-CSF for stimulating *proliferation of NFS60 cells. Data represent *means +/- SD for triplicate wells.

*IV. Summary of Phase I Experiments

* The Phase I studies have revealed that it is possible to create IgG-Fc fusions of EPO and G-CSF with complete *biological activity. IgG1-Fc fusions of both proteins had comparable biological activities to non-fused EPO and G-*CSF. As expected, the IgG-Fc fusion proteins were predominantly dimeric, presumably joined through disulfide *bonds in the hinge regions of the IgG domains. The fact that these fusion proteins were fully active suggests that *both halves of the dimeric proteins are biologically active. The EPO-IgG4-Fc fusion protein also retained complete *biological activity, but the G-CSF-IgG4-Fc fusion protein appeared to have slightly reduced biological activity.

* The EPO-IgG1-C_H and G-CSF-IgG1-C_H fusion proteins also were potent growth factors *in vitro*, although their *specific activities were reduced 2-3-fold relative to the non-fused proteins. These proteins also were *predominantly dimeric. The reduced bioactivities of these proteins correlated with the presence of high molecular *weight, disulfide-linked aggregates detected by SDS-PAGE analysis. Since these aggregates were not detected in *the original conditioned media, we believe they formed during purification of the proteins. The aggregates were *observed only with the IgG-C_H constructs and all of the IgG1-C_H constructs displayed aggregates. The IgG-C_H *fusion proteins may be aggregating via the hydrophobic domain in the CH1 domain that normally packs against the *IgG light chain (Traunecker et al., 1989). Aggregation appears to be accompanied by disulfide bond formation, *possibly through the free cysteine in the CH2 domain that normally disulfide bonds with a cysteine in the IgG light *chain. We propose experiments for Phase II to test this hypothesis and potentially improve/restore bioactivties of *the IgG1-C_H fusion proteins to wild type.

*V. Phase II Experimental Design and Methods

* The Phase II experiments have several goals. The primary goal will be to demonstrate the superior performance *of the EPO-IgG and G-CSF-IgG fusion proteins relative to non-fused EPO and G-CSF in *in vivo* models. *Specifically, we propose to demonstrate increased circulating half-lives for the EPO- and G-CSF-IgG fusion *proteins and efficacy equal to, or superior to, that of non-fused EPO and G-CSF, even when the fusion proteins are *administered less frequently and in lower doses. We believe these results are key to our moving forward to the *next stage of commercial development of this technology in that it will allow us to negotiate a *licensing/development agreement with a pharmaceutical or biotechnology company capable of rapidly bringing *EPO-IgG and G-CSG-IgG fusion protein products to market. The initial *in vivo* experiments will test the EPO- *IgG1-Fc and G-CSF1-IgG1-Fc fusion proteins since these proteins can be produced in adequate amounts and retain *complete biological activity.

* A second aspect of the proposed research will be to examine the need for a flexible linker between the growth *factor domain and the IgG domain. We will attempt to eliminate or minimize this linker in an effort to create an * "all human" molecule. If fully active fusion proteins lacking a linker or with a minimal linker are identified, we *will perform additional *in vivo* experiments with best of these proteins.

*A third aspect of the Phase II research is to continue efforts begun during Phase I to construct fully active IgG-C_H *fusion proteins. We propose experiments to co-express IgG light chains to prevent aggregation of the fusion *proteins. If these experiments are successful, we will perform additional animal studies with the these proteins.

* A fourth aspect of the proposed research will be to develop processes for high level recombinant expression of *the best fusion protein development candidates in stably transformed mammalian cells and to develop processes for *purification and characterization of these recombinant proteins.

*I. Animal Experiments with IgG-Fc Fusion Proteins

*A. Pharmacokinetic Experiments

* We will collaborate with researchers at BolderPATH, Inc., a local contract pharmacology, toxicology and *pathology company, to perform pharmacokinetic studies of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins *to determine if the fusion proteins have longer circulating half-lives than the corresponding non-fused proteins. *BolderPATH, Inc., an unaffiliated company, is based at the University of Colorado, Boulder. The animal *experiments will be performed at The University of Colorado, Boulder Animal Facility, which is an accredited *animal research facility. The pharmacokinetic data will guide us in designing animal experiments to determine *dosing regimens to compare the efficiencies of EPO-IgG1-Fc and G-CSF-IgG1-Fc to wild-type EPO and G-CSF. *Both intravenous and subcutaneous pharmacokinetic data will be obtained. Terminal pharmacokinetic parameters *will be calculated from the intravenous delivery data. These experiments will be performed initially with the IgG1- *Fc fusion proteins and later with other promising development candidates such as modified versions of these *proteins or fully active IgG-C_H constructs, if we are able to create them. We expect to find that the fusion proteins *have significantly longer circulating half-lives than the non-fused proteins.

* For the intravenous delivery studies, rats (~350g) will receive an intravenous bolus injection (0.1 mg/kg) of the *IgG1-Fc fusion protein (EPO or G-CSF) or the corresponding non-fused protein (EPO or G-CSF) and circulating *levels of the proteins measured over the course of 144 h. Three rats will be used for each protein sample. Blood *samples will be drawn at 0, 0.08, 0.5, 1.5, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h following intravenous *administration. The large number of blood samples to be analyzed is necessitated by the fact that IgG fusion *proteins can have half-lives of several days in a rat (Richter et al., 1999). The control proteins, EPO and G-CSF, *are expected to have circulating half-lives of 2-3 h (Elliot and Byrne, 1995; Tanaka et al., 1991). Serum levels of *the test proteins will be quantitated by Bolder Biotechnology researchers using commercially available EPO and G- *CSF ELISA kits (R & D Systems). Because of the cost of the ELISAs, serial dilutions of each blood sample will be *analyzed initially in the *in vitro* bioassays to identify dilutions that will fall within the linear range of the ELISAs.

*(0.025 to 1.6 ng/ml for EPO and 0.04 to 2.5 ng/ml for G-CSF). We will perform initial titration experiments to *determine the relative sensitivity of the ELISA for detecting the IgG1-Fc fusion proteins and the corresponding *non-fused proteins. This experiment will require 105 μg of each protein.

* The subcutaneous delivery studies will follow the same protocol as the intravenous studies except for the route *of delivery. Serum levels of the test proteins will be quantitated by ELISA as described above. This experiment *will require 105 μg of each protein.

***B. Animal Efficacy Models**

* Initially, we will determine *in vivo* efficacy of EPO-IgG1-Fc and G-CSF-IgG1-Fc in normal rats and mice since *these models are easier to perform. These studies will allow us to determine proper doses and dosing schedules. *Subsequently, we will determine efficacy of the proteins in appropriate disease models – anemia for EPO-IgG1-Fc *and neutropenia for G-CSF-IgG1-Fc. We will collaborate with researchers at BolderPATH, Inc. to perform the *animal studies, which will be performed at the University of Colorado, Boulder Animal Facility. BolderPATH has *experience in performing these bioassays. We expect to find that the IgG fusion proteins produce results equal or *superior to the non-fused proteins, but can achieve these results with less frequent dosing. We expect to find that *the IgG fusion proteins are more efficacious than the non-fused proteins when both are administered using the less *frequent dosing schedules.

* The pharmacokinetic experiments will provide guidance in deciding dosing schedules for the IgG1 fusion *proteins to be used for the animal studies. From published results with other IgG-Fc fusion proteins (Richter et al., *1999; Zeng et al., 1995) we expect the fusion protein will be effective when administered every other day or every *third day and possibly less often, e.g. a single injection. Because rodents metabolize proteins faster than humans * (Mordenti et al., 1991), dosing every other day or every third day in a rodent is roughly equivalent to dosing every *week or every other week in a human. The dosing schedules may have to be modified depending upon the results *of the pharmacokinetic studies and initial animal efficacy results. The dose of protein administered per injection to *the rodents also may have to be modified based upon the results of the pharmacokinetic experiments and initial *animal efficacy results. We have budgeted monies for experiments to determine whether the fusion proteins are *effective when administered every other day (EOD) and every third day (ETD).

***1. EPO Animal Efficacy Models**

* We will compare the *in vivo* efficacy of EPO-IgG1-Fc fusion proteins (and later other EPO-IgG fusion proteins) *to wild type EPO in stimulating increases increases in hematocrit and erythropoiesis in normal rats. Sprague-*Dawley rats (~200g) will be purchased from a commercial supplier such as Charles River (Wilmington, MA). *Previous studies have shown that administration of 100 IU/kg (approximately 800 ng/kg) of rEPO once per day * (160 ng SID / 200 g rat) by subcutaneous injection gives a significant increase in hematocrit and erythropoiesis in *rodents (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998; Sykowski et al., 1998). Groups of 5 rats *will receive subcutaneous injections of rEPO, EPO-IgG1-Fc or placebo (vehicle solution) at specified intervals for *up to five days. The highest dose of EPO-IgG1-Fc will be a molar equivalent rEPO. We will test a wide range of *EPO-IgG1-Fc doses (625-fold variation) for these initial experiments to increase the likelihood that one of the *doses will be effective. It is possible that administration of too much EPO-IgG1-Fc will impede erythropoiesis due *to toxicity. Control rats will receive vehicle solution only. Additional control groups will receive rEPO (160 *ng/SID for 5 days) and 160 ng rEPO using the same dosing regimen as EPO-IgG1-Fc. On day 6 the animals will be *sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis. *Hematopoietic tissues (liver and spleen) will be collected, weighed and fixed in formalin for histopathologic *analyses to look for evidence of increased erythropoiesis. Bone marrow will be removed from various long bones *and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased *erythropoiesis. Comparisons between groups will be made using a Students T test for single comparisons and one-*way analysis of variance for multiple comparisons. $P < 0.05$ will be considered significant.

* We expect to find that daily injections of rEPO stimulate increases in hematocrit and erythropoiesis in the rats, *whereas less frequent administration of the same dose of rEPO does not, or does so to a lesser extent. We expect to *observe dose-dependent increases in these parameters in the EPO-IgG-Fc-treated animals. We expect to observe *greater increases in these parameters in the EPO-IgG1-Fc-treated animals than in animals treated with EPO using *the less frequent dosing schedules. We also expect to find that significantly less EPO-IgG1-Fc is required to *achieve the same increases in these parameters obtained with daily injections of EPO.

* We will consider performing additional experiments with less frequent dosing, e.g., a single injection, if results *from the EOD nad ETD experiments are promising.

*EPO Experiment 1 – Normal Rats – Every Other Day Dosing: Rats will receive injections every other day *(EOD), i.e. on days 1, 3 and 5, for a total of three injections. Total amount of rEPO and EPO-IgG1-Fc required for *Experiment 1 is 6.4 μg and 8 μg, respectively.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (EOD)	5	0
2	EPO (160 ng SID)	5	4.0 μg
3	EPO (160 ng EOD)	5	2.4 μg
4	EPO-IgG1-Fc (0.64 ng EOD)	5	0.0096 μg
5	EPO-IgG1-Fc (3.2 ng EOD)	5	0.048 μg
6	EPO-IgG1-Fc (16 ng EOD)	5	0.24 μg
7	EPO-IgG1-Fc (80 ng EOD)	5	1.2 μg
8	EPO-IgG1-Fc (400 ng EOD)	5	6.0 μg

*EPO Experiment 2 – Normal Rats – Every Third Day Dosing: Rats will receive injections every third day *(ETD), i.e., on days 1 and 4, for a total of two injections. Total amount of rEPO and EPO-IgG1-Fc required for *Experiment 2 is 5.6 μg and 5 μg, respectively.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (ETD)	5	0
2	EPO (160 ng SID)	5	4.0 μg
3	EPO (160 ng ETD)	5	1.6 μg
4	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0064μg
5	EPO-IgG1-Fc (3.2 ng ETD)	5	0.032 μg
6	EPO-IgG1-Fc (16 ng ETD)	5	0.16 μg
7	EPO-IgG1-Fc (80 ng ETD)	5	0.8 μg
8	EPO-IgG1-Fc (400 ng ETD)	5	4.0 μg

*EPO Experiment 3 - Rat Anemia Model

* Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced anemia and has direct *relevance to the human clinical setting. rEPO reverses the anemia in this model when administered at daily doses *of 100 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). If EPO-IgG-Fc is capable of *increasing hematocrit levels in normal rats we expect it also will be effective at reversing anemia in this model. *The dosing schedule for EPO-IgG-Fc to be used in this experiment will be the one that worked best in the normal *rat experiments. The experimental protocol outlined below assumes EPO-IgG-Fc will be effective when *administered ETD, but this can be altered based upon results of experiments with normal rats. Sprague-Dawley *rats (~200g) will be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to induce anemia and *randomized to various treatment groups. The dosing schedule and amounts of protein injected per rat will be as *described for the ETD normal rat experiments described above. Rats will receive injections of EPO-IgG-Fc, rEPO *or saline on days 1, 4 and 7, for a total of three injections. One control group of rats will receive daily *subcutaneous injections of rEPO (100 Units/kg). Another control group will not receive the initial Cisplatin *injection but will receive ETD injections of saline. On day 9 the rats will be sacrificed and blood and tissue *samples obtained for comprehensive CBC and histopathology analyses. The amount of rEPO and EPO-IgG-Fc *required for this experiment is 9 μg and 7.5 μg, respectively.

Group	Cisplatin Treatment	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	-	Vehicle solution (ETD)	5	-
2	+	Vehicle solution (ETD)	5	-
3	+	EPO (160 ng SID)	5	6.4 µg
4	+	EPO (160 ng ETD)	5	2.4 µg
5	+	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0096µg
6	+	EPO-IgG1-Fc (3.2 ng ETD)	5	0.048 µg
7	+	EPO-IgG1-Fc (16 ng ETD)	5	0.24 µg
8	+	EPO-IgG1-Fc (80 ng ETD)	5	1.2 µg
9	+	EPO-IgG1-Fc (400 ng ETD)	5	6.0 µg

*C. Prepare Recombinant Proteins for Pharmacokinetic and Animal Efficacy Experiments

*1. EPO-IgG1-Fc and G-CSF-IgG1-Fc Fusion Proteins

* The total amounts of the IgG1-Fc fusion proteins required for the pharmacokinetic and animal efficacy *experiments described above are 250 μg EPO-IgG1-Fc and 500 μg G-CSF-IgG1-Fc. Using the COS cell transient *expression system and Protein A affinity column, we purified ~ 250 μg EPO-IgG1-Fc and ~125 μg of G-CSF-*IgG1-Fc from ~300 ml of conditioned medium collected by repeated harvests of seven T75 flasks. In order to *obtain animal data for pharmacokinetics and efficacy of these molecules as soon as possible, we will scale up the *COS cell transient expression system and Protein A affinity purification to produce sufficient material for the *proposed *in vivo* experiments. Ultimately, we will ultimately want to express these fusion proteins in stably

*transformed mammalian cell lines such as CHO cells and these plans are described below. However, development *of stable mammalian cell expression systems such as CHO cells typically employs multiple rounds of gene *amplification to achieve high level expression of heterologous genes and this process can be time consuming. *Therefore for the initial Phase II studies, we will produce the IgG1-Fc fusion proteins using the procedures *described in the Phase I summary for transfections and harvesting of conditioned media.

* For EPO-IgG1-Fc we will repeat the expression and purification of this protein from conditioned medium *collected from 7 T75 culture flasks, which which should yield ~250 μg of protein. We still have in hand >100 μg *of the EPO-IgG1-Fc protein that was purified during Phase I. Thus, one small (7 X T75) preparation should *provide more than enough material for the initial Phase II *in vivo* experiments.

* During Phase I we purified only 122 μg of G-CSF-IgG1-Fc from seven T75 culture flasks. For Phase II we plan *to scale-up the preparation ~ 7-fold. We will use 24 T150 flasks for production of conditioned medium. Assuming *results similar to those seen in Phase I, we project a recovery of ~850 μg which would be adequate. If needed, we *will repeat the 24 X T150 scale preparation. Pooled material from two preps on this scale should provide sufficient *material for the initial Phase II *in vivo* experiments.

***2. Non-Fused EPO and G-CSF Control Proteins**

* We will need to purify 250 μg rEPO and 450 μg rG-CSF for the pharmacokinetic and initial animal efficacy *experiments described above. The small amount of EPO required will allow us to produce this protein by transient *transfection of COS cells. G-CSF will be produced in transiently transfected COS cells or bacteria.

* For expression of rEPO in transiently transfected COS cells. We will modify the 3' end of *pCDNA3.1(+)::EPOfus (described above) to add DNA sequences encoding gly-gly-ser-asp-tyr-lys-asp-asp-asp-*asp-lys followed by a translational stop codon. We will add this sequence as a synthetic ~40 bp double-stranded *oligognucleeotide with *Bam* HI – *Eco* RI ends to *Bam* HI – *Eco* RI cut pCDNA3.1(+)::EPOfus. This will generate *an EPO construct with a carboxyterminal fusion of the 7 amino acid flexible linker present in the IgG fusions and *the "FLAG" epitope. This construct will be expressed in COS cells. The FLAG epitope will allow use an affinity *column based on the anti-FLAG monoclonal antibody M2 to purify this "FLAG-tagged" EPO. Using a *baculovirus expression vector, we have expressed the same "FLAG-tagged" EPO protein in insect cells and *purified it to homogeneity with an anti-FLAG monoclonal antibody M2 affinity column. This protein was fully *active *in vitro* and we have greater than 200 μg of the purified protein in hand. We prefer to use COS cell *expressed EPO as a control for EPO-IgG1-Fc produced in COS cells to avoid differences in protein glycosylation *that result from insect *versus* mammalian expression systems. However the baculovirus-produced material is *available as backup if we encounter unexpected problems with COS cell expression of EPO. We will assess the *level of EPO expression in transiently infected COS cells using an EPO ELISA and then scale the expression *system to an appropriate level to generate sufficient material (~250 μg) for the Phase II *in vivo* experiments.

* We have cloned and expressed human G-CSF in *E coli* as a protein secreted to the periplasm. We are currently *purifying this molecule and have not as yet determined its *in vitro* bioactivity. If the protein is fully active, we *should be able to use this system to purify sufficient *E. coli*-derived rG-CSF (~500 μg) for use in the *in vivo* *studies. Naturally occurring G-CSF contains one O-linked glycosylation site and no N-linked glycosylation sites *so that the distinction between *E coli* and COS derived proteins will be slight. Moreover the current G-CSF *commercial product, Neupogen, is produced in *E coli*. If for unforeseen reasons we are unable to purify active G-*CSF from *E coli*, we will modify the pCDNA3.1(+)::G-CSFfus plasmid to express a FLAG-tagged G-CSF via the *same modifications described above for EPO. We do not know if a "FLAG-tagged" G-CSF will be fully active, *but this seems likely since the carboxyterminal fusion of the much larger IgG1-Fc protein did not interfere with G-*CSF bioactivity. If we use COS cell expression, we will assess the level of G-CSF expression in transiently *transfected COS cells using a G-CSF ELISA and then scale the expression system to an appropriate level to *generate sufficient material (~500 μg) for the Phase II *in vivo* experiments.

***II. Create Stably Transfected Mammalian Cell Lines Expressing the GF-IgG Fusion Proteins**

* Transient expression of the the GF-IgG fusion proteins using the COS cell expression system will provide the *quickest route to *in vivo* testing of IgG fusion proteins for pharmacokinetics and animal efficacy. Ultimately, *though, we expect to manufacture the proteins in stably transformed mammalian cell lines. Stably transfected *CHO (Chinese Hamster Ovary) cell lines are widely used for recombinant protein expression (Geisse et al. 1996; *Trill et al. 1995). High level expression of chromosomally integrated heterologous genes in CHO cells can be *achieved by gene amplification. Typically the gene of interest is linked to a marker gene for which amplification is *selectable. A number of genes that provide selections for amplification have been described (Kaufman 1990) but

*murine dihydrofolate reductase (dhfr) is perhaps the most frequently employed. Amplification of this gene confers *resistance to the folate analog methotrexate (MTX) and level of resistance is proportional to the dhfr gene copy *number (Alt et al. 1978). Utility of this selection is enhanced by the availability of mutant CHO cell lines that are *deficient in dhfr (Urlaub and Chasin, 1980). Typically a plasmid carrying the gene of interest and the murine dhfr *gene is tranfected into a dhfr$^-$ CHO cell line and stable transformants selected using the dhfr$^+$ phenotype or by *resistance to a lethal drug such as G418 (geneticin sulfate), which is conferred by a plasmid-borne resistance gene. *Neomycin phosphotransferase (NPT) is the most common gene used to confer resistance to G418. Subsequent *multiple rounds of selection for resistance to increasing levels of MTX results in amplification of the plasmid-*derived dhfr gene. In a fraction of the MTX resistant clones the level of expression of the gene of interest is *correspondingly increased.

* The procedures for tranfection, selection and amplification in CHO cells are well described in the literature and *have been used to express high levels of a number of immunoglobulin fusion molecules (Chamow and Ashkenazi *1996) of a variety of heterologous proteins (Geise, 1996; Kaufman, 1990). Amplification can typically yield cell *lines producing 1-5 ug/ml of a desired recombinant protein in T flasks and 5 to 10-fold higher levels in roller bottle *culture. Immunglobulins such as monoclonal antibodies typically are expressed at relatively high levels in CHO *cells (Trill et al 1995). Expression of our fusion proteins in COS cells is on the order of ~ 1 ug / ml per 72 h, which *is in the normal range for immunoglobulins such as monoclonal antibodies expressed in COS cells (Trill et al *1995). Hopefully this will translate into robust expression our our fusion proteins in CHO cells as well, although *COS cell results are not always predictive of results from expression in stable cell lines (Trill et al 1995).

* We will construct expression vectors for EPO-IgG1-Fc and G-CSF-IgG1-Fc that will incorporate the murine *dhfr gene into the commercially available pCDNA3.1 expression vector (Invitrogen), which includes the NPT *gene. The murine dhfr expression vector pdhfr2.9 is available from ATCC (# 37165). This plasmid expresses *mouse dihydrofolate reductase in eukaryotic cell lines. The dhfr gene is selectable in dhfr$^-$ CHO cell lines and can *be amplified by standard selections for MTX resistance (Crouse et al, 1983). The dhfr coding sequence can be *excised from pdhfr2.9 as a ~ 900 bp *Bgl* II fragment, which we will clone into the unique *Bam* HI site of the *polylinker of the expression vector pREP4 (Invitrogen). This construct will position the dhfr coding sequence *downstream of the strong RSV promoter, which is known to function in CHO cells (Trill et al, 1995) and upstream *of a polyadenylation site deived from SV40. This dhfr expression cassette can then be conveniently excised from *pREP4 as a *Sal* I fragment since *Sal* I sites closely flank the promoter and polyA addition site. Using *olignucleotide linkers this *Sal* I fragment will be cloned into the unique *Bgl* II site of pCDNA3.1. The EPO-IgG1-*Fc and G-CSF-IgG1-Fc genes will subsequently be cloned into the *Hind* III – *Xba* I. sites of pCDNA3.1 polylinker *region under control of the CMV promoter.

* Endotoxin free plasmid DNAs will be used to transfect dhfr$^-$ CHO cells. We will obtain a dhfr$^-$ CHO line, either *CHO K1 DUKX B11 from L. Chasin at Columbia University or CHO duk$^-$ from the ATCC (# CRL-9096). Cells *will be cultured in F12/DMEM medim supplemented with 10% FCS, glutamine, glycine, hypoxanthine, and *thymidine (Lucas et al., 199X). Transfections will be carried out with LipofectAMINE (Gibco BRL) using *protocols similar to those used during Phase I. We will select for dhfr$^+$ transfectants in F12/DMEM supplelmented *with 7% dialyzed FCS and lacking , glycine, hypoxanthine, and thymidine (Lucas et al., 199X). Alternatively, we *will select for G418 resistance and subsequently screen transfectants for the dhfr$^+$ phenotype. Dhfr$^+$ clones will be *expanded in selection medium and screened for EPO-IgG1-Fc or G-CSF-IgG1-Fc production by ELISA (R&D *Systems). Clones with the highest expression levels will be pooled and subjected to multiple rounds of selection *for MTX resistance at increaing drug concentration as described by Kaufman (1990). After each round of MTX *selection, a set of individual clones will be tested for EPO-IgG1-Fc and G-CSF-IgG1-Fc production.

***III. Eliminate or minimize the linker in the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusions.**

* We will determine the importance, in any, of the flexible peptide linker [ser-gly-gly-ser-gly-gly-ser] that fuses *the growth factor domain to the immunoglobulin domain. We included the linker in our initial constructs to *increase the likelihood that the fusions would be active. Typically, fusions to the IgG hinge region have not *employed such flexible linkers, as the hinge itself functions in this capacity (Chamow and Ashkenazi, 1996). In *principle there is no reason to include such a linker in a compound to be used in human subjects unless it is *important for optimal functioning of the molecule. To determine if the peptide linker is important for biological *activity of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins we will construct modified forms of these two *fusions that shorten (to two or four residues) or eliminate the linker.

* We will use PCR based "gene splicing by overlap extension" as described in Phase I to generate GF-IgG – Fc *fusions without a linker. We will generate PCR products consisting of the IgG1-Fc coding sequence with a short *5' extension, consisting of the 3' terminal ~ 15 bp of coding sequence of EPO or G-CSF fused directly to the hinge

*coding sequence. At the same time we will generate PCR products consisting of the EPO or G-CSF coding *sequences with a short 3' extension consisting of the first 15 bp of the hinge coding sequence fused directly to the *EPO or G-CSF coding sequence. The growth factor fragments and the IgG1-Fc fragments can then be spliced *together via PCR "Sewing" to generate direct fusions. These PCR products will be digested with appropriate *restriction enzymes to generate relatively small DNA segments that span the fusion point and which can be readily *cloned into similarly cut vectors pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc for sequence *confirmation and COS cell expression. Cloning these smaller DNA fragments will minimize the sequencing that *will need to be done to confirm the sequences of the direct fusions.

* To construct a di-peptide [ser-gly] linker, we will PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds *the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for *the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment will be digested with *Bsp* *EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and *pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the *linker [ser-gly-gly-ser-gly-gly-ser] so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker. *The sequence of the newly inserted ~ 250 bp *Bsp* EI – *Sac* II fragment will be verified.

* A similar procedure will be used to construct the 4 amino acid [ser-gly-gly-ser] linker. We will PCR the IgG1- *Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCGGATCC to the hinge coding sequence. The *GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly- *ser. This PCR fragment will be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly *cut pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these *plasmids occurs at the first gly-ser in the linker [ser-gly-gly-ser-gly-gly-ser] so the recombinants will contain the 4 *amino acid (ser-gly-gly-ser) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II peice will be verified.

* During Phase I we measured specific activities of the IgG fusion proteins using purified proteins. In retrospect, *we have found that combining bioactivity data from unpurified COS cell supernatants with quantitation of the IgG- *Fc fusion proteins in the COS cell supernatants by ELISA gives specific activity results essentially identical to *those obtained with the purified fusion proteins. This is the approach we will take to measure specific activities of *the new linker constructs. The linker variants will be transfected into COS cells using the small-scale 6-well plate *procedure described in the Phase I report. Concentrations of the IgG fusion proteins in the COS cell supernatants *will be measured using EPO and G-CSF ELISAs. Bioactivities of the supernatants will be measured using the *established bioassays described in the Phase I report. From these data we can determine specific activities of the *new fusion constructs. The original linker constructs will be analyzed in parallel for controls. Those linker *variants that appear to be fully active in this screen will be scaled up for purification and further characterization.

***IV. Improve Bioactivities of IgG1-C_H Fusion Proteins**

* The IgG-C_H fusion constructs analyzed during Phase I appear to aggregate during purification and the specific *activities of the fused growth factors were reduced ~ 2-3-fold as compared to the analogous Fc fusions. *Aggregation may be due to hydrophobic interactions involving the CH1 domain that normally interfaces with the *light chain. Coexpression of light chains may prevent aggregation. Most IgG fusions have employed the Fc *portion of the immunoglobulin genes and it has been suggested that secretion and assembly of such fusions in the *absence of light chain expression is improved by deletion of the CH1 domain (Chamow and Ashkenazi 1996). We *are aware of only two IgG-C_H fusions: CD4-IgG-C_H (Capon et al, 1989) and IL-2- IgG-C_H (Landolphi, 1994). *Most IgG fusion molecules have been constructed as research tools to identify and characterize receptor – ligand *interactions and the biological functions of these interacions (Chamow and Ashkenazi, 1996) and IgG-Fc fusions *typically function well for the these types of experiments. Our focus with these fusions is on extending *in vivo* half *life of the fused cytokine element and for this purpose complete heavy chain fusions, coexpresssed with the light *chain constant domain could potentially provide longer circulating half lives than the smaller Fc fusions. These *larger, more complex structures will more closely resemble bona fide IgG molecules and as such may have *circulating half lives closer to that of IgGs. All of the Fc fusion molecules for which there are *in vivo* data on *circulating half life (Richert et al., 1999; Zeng et al., 1995) fall short of the 21 day half-life of the IgG molecule *(Roitt et al., 1989). Therefore, during Phase II we will pursue active EPO and G-CSF fusions with IgG1-C_H by *cloning and coexpressing human light chains.

* The DNA sequences of human kappa and lambda light chains are known (Heiter et al., 1980). We will clone *the DNA sequences encoding the human kappa and / or lambda light chain constant (CL) regions by PCR *amplification from the human leukocyte single-stranded cDNA (Clontech) used to clone the IgG1 and IgG4 heavy *chain sequences during Phase I. We will verify the DNA sequences of the cloned CL domains and then use PCR *based mutagensis to modify the 5' and 3' ends for expression in mammalian cells in three different formats.

* 1. We will add a Kozak sequence and a secretion signal to the 5' end to enhance translational initiation and *direct the secretion of the light chain. We will add a translational stop codon to the 3' end of the sequence. *Appropriate cloning sites will be added to the 5' and 3' ends to allow cloning into the mammalian cell expression *vector pREP4 (Invitrogen) under control of the RSV promoter and preceding the SV40 derived polyA addition site. *This construct will be used to cotransfect COS cells along with pCDNA3.1(+) derivatives that express EPO-IgG-*C_H and G-CSF-IgG-C_H. If we prefer to express both light and heavy chains from a single construct, we will excise *the light chain sequence and the flanking promoter and polyA sites from pREP4 and clone this fragment into *pCDNA3.1(+). The EPO-IgG-C_H and G-CSF-IgG-C_H coding sequences could then cloned into the pCDNA3.1(+) *polylinker under control of the CMV promoter.

* 2. An alternative mode of light chain expression will be to modify the 5' end to add a portion of a flexible linker *sequence fused to the amino-terminus of the CL coding sequence and add a translational stop codon to the 3' end *of the sequence. Appropriate cloning sites will be added as well to the 5' and 3' ends to allow cloning as an in *frame fusion to the EPO and G-CSF genes cloned in the plasmids pCDNA3.1(+)::EPOfus and pCDNA3.1(+)::G-*CSFfus. We will cotransfect this plasmid into COS cells with plasmids that express EPO-IgG1-C_H and G-CSF-*IgG1-C_H. In this instance both heavy and light chains will contain growth factor fusions. The light and heavy *chains could be expressed from a single pCDNA3.1 construct as described above.

* 3. A third mode of "co-expression" would be to modify the 5' and 3' ends of the CL coding sequence to *incorporate portions of a flexible linker at both ends. By also incorporating appropriate cloning sites (*Bsp* EI and **Bam* HI) such a construct can be inserted into the Bsp EI and *Bam* HI sites within the flexible linkers of the EPO-*IgG-C_H and G-CSF-IgG-C_H fusions in pCDNA3.1(+). The resulting constructs would encode single polypeptide *[EPO]-[CL]-[IgG-C_H] and [G-CSF]-[CL]-[IgG-C_H] fusions. The fusion of the carboxy-terminus of the light chain *constant region to the amino-terminus of the heavy chain CH1 domain seems reasonable by analogy to single chain *Fv polypeptides. Flexible peptide linkers of the (ser-gly-gly) motif on the order 14 to 20 residues in length have *been used to fuse the carboxy-terminus of the light chain variable region to the amino-terminus of the heavy chain *variable domain (Stewart et al., 1995).

EXHIBIT M

Project No.
Book No.
TITLE 4/30/99 Transfec pBIST 180 Purification

6/8/99

Purify EPO-IgG1 Fc from CM. This transfection was Lg Scale
(10 T150 flasks from 4/30/99

1. Thaw CM. Vol = 950ml
Conc on YM30 membrane

St. vol	950ml	after conc ↓ to 150 ml → fr to clear ppt then pack on ice O/N
Conc ↓	90ml	Continued conc. on 6/9/99.
Rinse mem c	5ml	
Final vol	95ml	= 10X Conc.

2 Load onto Equilib. Prot. A Col 1ml HiTrap.
F/R ~0.8ml/min
Collect F/T as 1 frac

3 Wash c 20mM NaP; pH 7.0 10 col vol
F/R 1ml/min Collect as 1 frac

4 Elute c 0.1M Na citrate pH 3.0. Collect 0.5ml frac into 0.1ml
F/R 1ml/min 1M Tris pH 9.0

√ pH of frac. & Adj to ~ 7.0-7.5

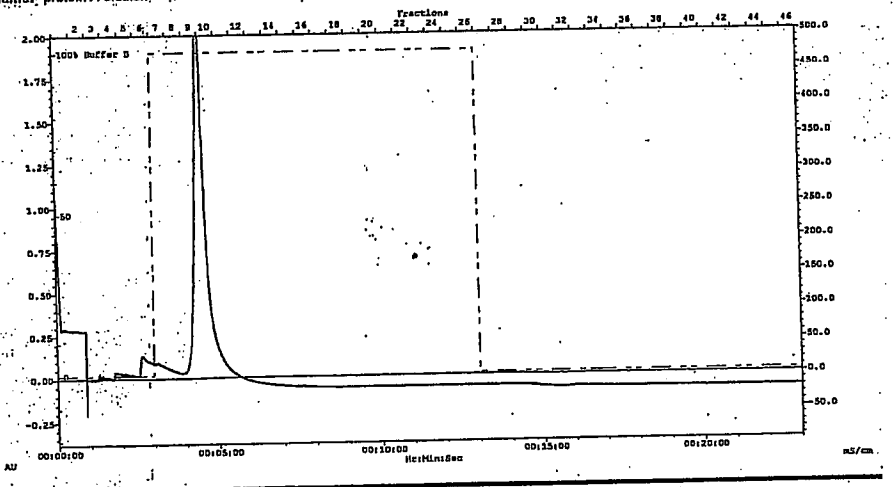
To Page No.

Witnessed & Understood by me, Mary S R Date 9/2/99
Invented by [signature] Date 6/16/99
Recorded by

Page No.

BioLogic Run Report Printed Date: 17-Jun-99

Method Name: protein A elution Run Name: Protein A col 4/30/99 transfec pB Run Date: 11:44:15 AM 06-09-99



Sample: 4/30/99 transfection pBBT 180 EPO-IgG1-Fc
Column: Protein A 1ml HI trap
Buffer A: 20 mM NaPi, pH7.0
Buffer B: 0.1 M NaCitrate pH 3
Operator: Darin

Flow Rate: 1.0
Gradient:
Chart Speed:
Fraction Size: 0.5
Run Description:

1

After reviewing gels. Pool #'s 9, 10 & 11 Vol = 1.8ml

Bradford Assay (p.109) → Results Conc is 382 µg/ml

Total protein ~ 687 µg

Visual gel p110

To

Witnessed & Understood by me,

Date 9/2/99

Invented by

Recorded by

Date 6/16/99

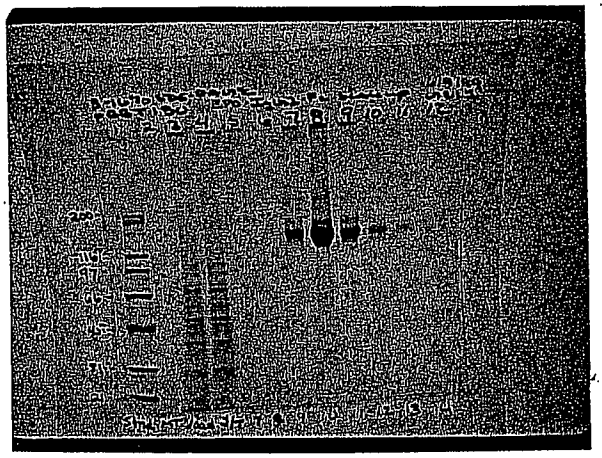
Project No._____

108 Book No._____ TITLE SDS. PAGE of Prot. A-Col pBB180

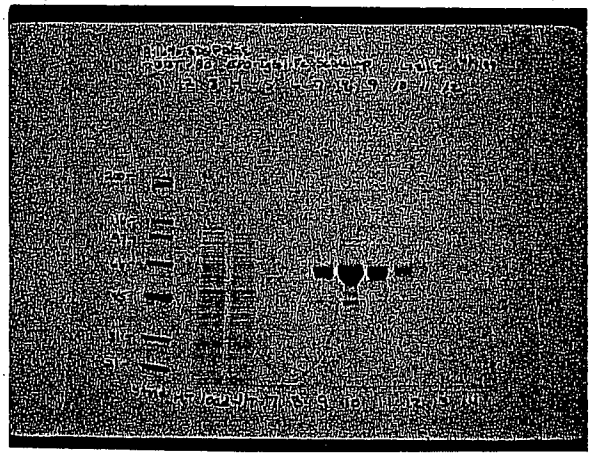
From Page No.___ 6/9/1

8-16% SDS. PAGE GEL 1 N.R. GEL 2 RED

Lane	Sample
1	Biorad std
2	mT
3	Col load
4	HT
5	JNS 7
6	8
7	9
8	10
9	11
10	12
11	13
12	14



Sample Prep
20µl Sample + 5µl SVSBM-5% ME
heat 100°C 5min load all
Run on GEL at ~140V C.V.



To Page No.___

Witnessed & Understood by me,	Date	Invented by	Date
Jay S	9/2/99		6/16/99
		Recorded by	

EXHIBIT N

Project No.

E 7/6-9/99 EPO BioAssay Book No. 175

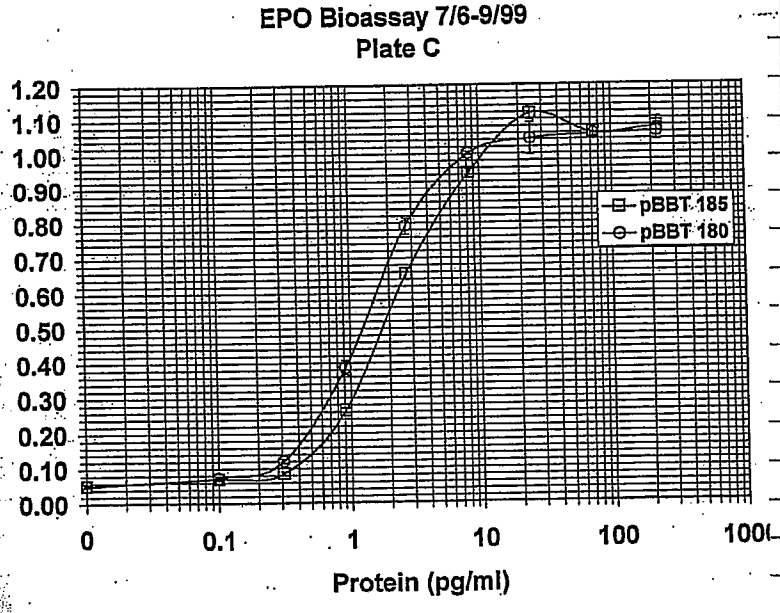


Plate C 7/9/99

pBBT 185

hi use: 222,74/24.7ng/ml = 1.084

low use: 0ng/ml = .052

1.084 - .052 x .052 = .568 = 12

2 EC50 = 2.1ng/ml

pBBT 180

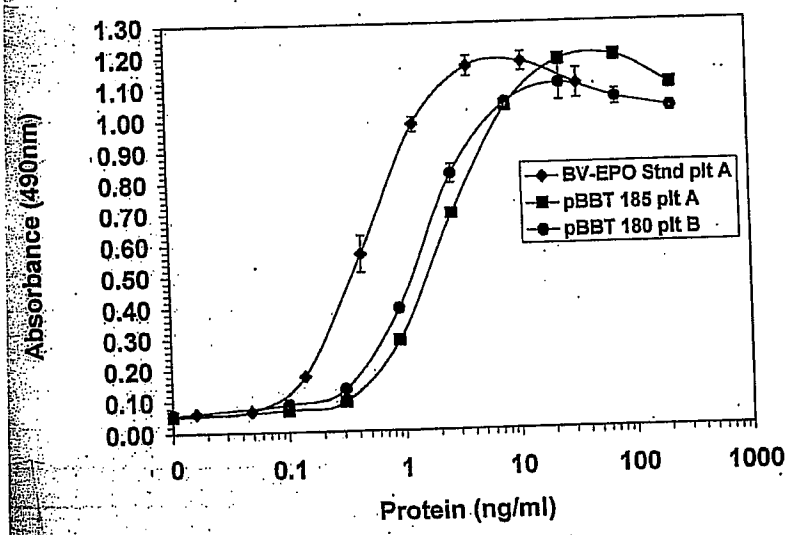
hi use: 222,74ng/ml = 1.058

low use 0ng/ml = .054

1.058 - .054 x .054 = .556 = 12

2

EC50 = 1.3ng/ml



EC50's

Sample	Plate	EC50
BV-EPO	A	.48ng/ml
	B	.48ng/ml
pBBT 185	A	2ng/ml
	C	2.1ng/ml
pBBT 180	B	1.3ng/m
	C	1.3 ng/m

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	7/15/99

EXHIBIT O

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)(2).

Docket Number	4152-3PROV	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)			
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Cox, III	George	N.	678 West Willow Street Louisville, Colorado 80027
Doherty	Daniel	H.	719 Ithaca Drive Boulder, Colorado 80303

TITLE OF THE INVENTION (280 characters max)
"IMMUNOGLOBULIN FUSION PROTEINS"

CORRESPONDENCE ADDRESS					
Theresa A. Brown Sheridan Ross P.C. 1700 Lincoln Street, Suite 3500 Denver					
STATE	Colorado	ZIP CODE	80203	COUNTRY	United States of America

ENCLOSED APPLICATION PARTS (check all that apply)						
X	Specification	Number of Pages	66	X	Small Entity Statement	
X	Drawing(s)	Number of Sheets	1		Other (specify)	

METHOD OF PAYMENT (check all that apply)				
X	A check or money order is enclosed to cover the filing fees		PROVISIONAL FILING FEE AMOUNT ($)	$75.00
X	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	19-1970		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

_____.

Theresa A. Brown
Registration No. 32,547

"EXPRESS MAIL" MAILING LABEL NUMBER: EL323946529US
DATE OF DEPOSIT: July 13, 1999

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.

TYPED OR PRINTED NAME: CONSTANCE ROBNETT

SIGNATURE: Constance Robnett

Immunoglobulin Fusion Proteins

Field of the Invention

This invention relates generally to methods for constructing proteins and more specifically to methods for constructing recombinant IgG fusion proteins.

Background of the Invention

Prolonging the circulating half-lives of protein pharmaceuticals is of interest to patients and healthcare providers. Long acting protein therapeutics will require less frequent injections and should be effective at lower doses than proteins with shorter circulating half-lives. It is known that increasing the effective size of a protein can increase its circulating half-life by preventing removal of the protein by the kidney (Knauf et al., 1988; Mahmood, 1998). One method that can be used to increase the effective size of a protein is to use recombinant DNA technology to covalently fuse the protein of interest to a second protein. The larger fusion protein often has a longer circulating half-life than the non-fused protein (Capon et al., 1989; Zeng et al., 1995). One class of proteins that has been used frequently to create fusion proteins is immunoglobulins (Ig), which are major components of blood. Immunoglobulins occur in various classes known as IgG, IgM, IgA, IgD, and IgE (Roitt et al., 1989). Human IgGs can be further divided into various types known as IgG1, IgG2, IgG3 and IgG4, which are products of distinct genes. IgG1 is the most common immunoglobulin in serum (70% of total IgG) and has a serum half-life of 21 days (Capon et al., 1989; Roitt et al., 1989). Although less abundant, IgG4 also has a long circulating half-life of 21 days (Roitt et al., 1989)

Human IgGs have a multidomain structure, comprising two light chains disulfide-bonded to two heavy chains (reviewed in Roitt et al., 1989). Each light chain and each heavy chain contains a variable region joined to a constant region. The variable regions are located at the N-

terminal ends of the light and heavy chains. The heavy chain constant region is further divided into CH1, Hinge, CH2 and CH3 domains. The CH1, CH2 and CH3 domains are discreet domains that fold into a characteristic structure. The Hinge region is a region of considerable flexibility. Flexibility of the hinge can vary depending upon the IgG isotype (Oi et al., 1984; Dangl et al., 1989). IgG heavy chains normally form disulfide-linked dimers through cysteine residues located in the Hinge region. The various heavy chain domains are encoded by different exons in the IgG genes (Ellison et al., 1981; 1982).

Proteins have been fused to the heavy chain constant region of IgGs at the junction of the variable and constant regions (thus containing the CH1-Hinge-CH2-CH3 domains - referred to herein as the IgG-C_H fusions) at the junction of the CH1 and Hinge domains (thus containing the Hinge-CH2-CH3 domains - referred to herein as IgG-Fc fusions), and at the C-terminus of the IgG heavy chain (referred to herein as IgG-C-terminal fusions).

IgG fusion proteins have been created most often with the extracellular domains of cell surface receptors (reviewed in Chamow and Ashkenaki, 1996). Examples of extracellular domains of cell surface receptors that have been joined using recombinant DNA technology to the C_H or Fc domains of human or mouse IgGs include CD4 (Capon et al., 1989), tumor necrosis factor receptors (Mohler et al., 1993), CTLA4 (Linsley et al., 1991a), CD80 (Linsley et al., 1991b), and CD86 (Morton et al., 1996). Extracellular domains of receptors evolved to function when fused to other amino acids, i.e., the transmembrane and intracellular domains of the receptor; therefore it is not surprising that extracellular domains retain their ligand binding properties when fused to other protein domains such as IgG domains. Despite this, differences in ligand binding properties have been noted for certain extracellular domains. For example, a fusion protein comprised of the extracellular domain of CD4 to human IgG1-C_H had 2-fold reduced affinity for the CD4 ligand gp120 than non-fused CD4 (Capon et al., 1989).

There are significantly fewer examples of proteins that are normally soluble, e.g., growth factors and cytokines, etc, which have been fused to IgG domains and retained full biological activity. Soluble proteins did not evolve to function when fused to other proteins and there is no reason to expect them to retain biological activity when fused to other proteins. In fact, in the majority of the published examples, biological activity of the fused cytokine/growth factor was significantly reduced relative to the non-fused cytokine/growth factor (see below). Whether or not the cytokine/growth factor will function properly when fused to another protein will depend upon many factors, including whether the amino-terminus or carboxy-terminus of the cytokine/growth factor is exposed on the surface of the protein, whether these regions are important for biological activity of the cytokine/growth factor and whether the cytokine/growth factor is able to fold properly when fused to another protein. By their very nature, such factors will be highly protein-specific. Results with the few growth factor/cytokine fusion proteins that have been studied have shown how protein-specific biological activity of the fusion protein can be. In the majority of cases, biological activity of the fused growth factor/cytokine is severely reduced, whereas, in the minority of cases full biological activity of the growth factor/cytokine is retained. In one case where biological activity of the fusion protein was significantly reduced, modifying the amino acids at the junction between the cytokine/growth factor and the IgG domain resulted in a fusion protein with improved biological activity. This same modification did not improve biological activity of a second cytokine fused to the same IgG domain (see below).

Growth factors that have been fused to IgGs include keratinocyte growth factor (KGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-I). A KGF-mouse IgG1-Fc fusion protein was created by LaRochelle et al. (1995). On a molar basis, the fusion protein was 4-5-fold less active than KGF in stimulating proliferation of Balb/MK cells in an *in vitro*

bioassay. The KGF-IgG-Fc fusion also had approximately 10-fold lower affinity for the KGF receptor on cells than did KGF. A fibroblast growth factor-human IgG-Fc fusion was constructed by Dikov et al. (1998). On a molar basis the FGF- IgG1-Fc fusion protein was approximately 3-fold less active than FGF in *in vitro* assays in stimulating DNA synthesis in NIH 3T3 cells. Shin and Morrison (1990) fused IGF-I to the C-terminus of IgG and found that the IGF-I-IgG C-terminal fusion protein had less than 1% of the *in vitro* biological activity of IGF-I.

Examples of cytokines that have been fused to IgG domains include IL-2, IL-4, IL-10 and GM-CSF. Landolphi (1991; 1994) described an IL-2-IgG1-C_H fusion protein, which included an extra amino acid, serine, between the C-terminus of IL-2 and the N-terminus of the IgG-C_H domain. The IL-2-IgG1-C_H fusion protein was as active, on a molar basis, in *in vitro* bioassays as IL-2, but no details were provided as to how protein concentrations were quantitated (Landolphi, 1991; 1994). Zeng et al. (1995) fused mouse IL-10 directly to the Fc region of mouse IgG2a; however the first amino acid of the Fc hinge region was changed from Glu to Asp. Zeng et al (1995) reported that the IL-10-IgG2a fusion protein was fully active in *in vitro* bioassays; however, only two concentrations of the fusion protein were studied, both of which were saturating. These high protein concentrations would have allowed only major differences (e.g., 100-fold) in bioactivities between the IL-10-mouse IgG2a fusion protein and IL-10 to be detected. To detect smaller differences in bioactivities, one needs to analyze serial dilutions of the proteins in *in vitro* bioassays and calculate EC_{50}s (the amount of protein required for half-maximal stimulation). EC_{50}s of the IL-10-IgG2a and IL-10 were not reported by Zheng et al. (1995). Chen et al.(1994) also constructed an IL-2-IgG fusion protein and reported that this fusion protein was fully active. Gillies et al. (1993) also reported creating a fully active IL-2 fusion protein comprising IL-2 fused to the C-terminus of an antiganglioside IgG antibody.

Unexpectedly, Gillies et al. (1993) found that a fusion between the same antibody and GM-CSF displayed only 20% of wild type GM-CSF bioactivity. Chen et al. (1994) were able to create a fully active IgG-C-terminal- GM-CSF fusion protein by inserting four amino acids between the antibody molecule and GM-CSF. Unexpectedly, they reported that fusion of IL-4 to the same antibody using the same four amino acid linker resulted in an IL-4 protein with 25-fold reduced biological activity (Chen et al., 1994).

Qiu et al. (1998) described homodimeric erythropoietin (EPO) proteins in which two EPO proteins were fused together using flexible peptide linkers of 3-7 glycine residues. The peptide linker joined the C-terminus of one EPO protein to the N-terminus of the second EPO protein. In vitro bioactivities of the fusion proteins were significantly reduced (at least 4-10-fold) relative to wild type EPO (Qiu et al., 1998).

It appears that the amino acids at the junction between the growth factor/cytokine domain and the IgG domain can have a profound influence on the biological activity of the fused growth factor/cytokine.

In work described herein, the inventors disclose IgG-Fc fusion proteins of erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) that have biological activities, on a molar basis, comparable to non-fused (EPO) and G-CSF. In contrast, the inventors discovered that IgG1-C_H fusions of EPO and G-CSF had reduced specific activities (2-3-fold) relative to the non-fused proteins. The inventors also found that identical IgG-Fc and IgG1-C_H fusion constructs using growth hormone (GH) had significantly reduced (4 to 17-fold) biological activity relative to non-fused GH. These results provide further evidence as to the unpredictability of IgG fusion protein bioactivities.

IL-2, IL-4, GM-CSF, GH, EPO and G-CSF have nearly identical structures, comprising four alpha helices joined by loops (reviewed in Mott and Campbell, 1995). The characteristic

structure of these proteins is known as the four helix bundle. The four helix bundle structure is shared by a large number of other cytokines and growth factors, including GH, EPO, TPO, G-CSF, GM-CSF, M-CSF, IL-2, IL-3, IL-4, IL-5 IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, alpha, beta and gamma interferon, CNTF, LIF, and oncostatin M (reviewed in Mott and Campbell, 1995). The fact that these proteins share a conserved structure makes it all the more surprising that bioactivities of the proteins are affected so differently when they were fused to other proteins such as IgGs.

The idea of creating IgG fusion proteins with other cytokines and growth factors, and in particular EPO and G-CSF, are contemplated in patent applications EP 0 464 533 A and WO99/02709. The possibility of creating IgG fusion proteins with cytokines and growth factors also is disclosed by Landolphi (1994). None of these patents or patent applications present any data regarding expression, purification and bioactivities of the contemplated EPO-IgG and G-CSF-IgG fusion proteins. Landolphi (1994) presents bioactivity data for an IL-2-IgG1-C_H fusion protein, but not for any other IgG fusion protein. The EPO-IgG1-Fc fusion protein contemplated by EP 0 464 533 A would contain the two amino acid linker, ProGlu, between Asp165 of the EPO coding sequence and the beginning of the IgG1-Fc hinge region. The C-terminal amino acid in EPO, Arg166 would be deleted. EP 0 464 533 A does not provide any information as to how the G-CSF coding region would be joined to the IgG1-Fc hinge region. Bioactivity data for neither protein is provided in EP 0 464 533. WO 99/02709 discloses construction of an EPO-mouse IgG2a-Fc fusion protein, but not an EPO-human IgG fusion protein. Bioactivity data for the EPO-mouse IgG2a-Fc fusion protein are not presented. WO 99/02709 also does not provide details as to the source of the EPO cDNA or human IgG genes used to construct the contemplated fusion proteins or the precise amino acids used to join EPO to the IgG domain. The reference cited in WO 99/02709 (Steurer et al., 1995) also does not provide this information.

Accordingly, WO 99/02709 does not adequately teach how EPO should be joined to an IgG domain. WO 99/02709 postulates that a flexible peptide linker of 0-20 amino acids can be used to join EPO to the IgG domain. The amino acids to be used to create the flexible linkers are not specified by WO 99/02709. Qiu et al. (1998) reported that EPO fusion proteins joined by flexible linkers of 3-7 glycine residues have significantly reduced biological activities (4-10-fold) relative to wild type EPO. Thus it is clear from the literature that the precise amino acids used to construct the linker fusing the cytokine/growth factor to the second protein are critical for determining bioactivity of the fusion protein. Since a peptide linker of 20 amino acids can have 20^{20} possible sequences (since there are 20 different amino acid possibilities for each position in the sequence) it would require undo experimentation to test all possible linker sequence permutations to identify a linker that allows complete bioactivity of the cytokine/fusion protein to be retained.

In work described herein, the inventors discovered that bioactivities of fusion proteins also can vary depending upon the isotype of the IgG domain. This result was not predicted from the literature. The inventors postulate that the bioactivity variability thry observed may relate to the flexibility of the hinge region in the IgG domain used to construct the fusion proteins. Based upon these findings, the inventors believe that bioactivity of an EPO- mouse IgG fusion protein cannot be used to predict bioactivity of an EPO- human IgG fusion protein.

Brief Description of the Figure.

Figure 1 shows reduced and non-reduced SDS-PAGE analysis of purified GH-, G-CSF- and EPO-IgG-fusion proteins. Panel A shows purified Growth Hormone (GH)-IgG fusion proteins. Lane 1, molecular weight standards; Lanes 2 & 3 are bovine IgG standard at 1 & 2μg respectively, reduced; Lanes 4,5 & 6 are GH-IgG1-C_H, GH-IgG1-Fc, and GH-IgG4-Fc

respectively, reduced; Lane 8, 2μg bovine IgG standard non-reduced; Lanes 9,10 & 11, are identical to lanes 4,5 & 6 except non-reduced. Panel B shows purified G-CSF-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are G-CSF-IgG1-C_H, G-CSF-IgG1-Fc and G-CSF-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced. Panel C shows purified EPO-IgG fusion proteins. Lanes 1,2,3 & 8 are identical to those in Panel A; Lanes 4,5 & 6 are EPO-IgG1-C_H , EPO-IgG1-Fc and EPO-IgG4-Fc respectively, reduced; Lanes 9,10 & 11 are identical to lanes 4,5 & 6 except non-reduced.

Detailed Description of the Invention

The present invention provides methods for constructing novel EPO-IgG fusion proteins and G-CSF-IgG fusion proteins that possess *in vitro* biological activities equal to or within 2-fold, on a molar basis, the biological activities of non-fused EPO and G-CSF. The invention discloses other methods for constructing EPO and G-CSF-IgG fusion proteins that possess *in vitro* biological activities within 4-fold of non-fused EPO and G-CSF. The invention also discloses methods for constructing novel GH-IgG fusion proteins that possess specific *in vitro* bioactivities within 4-17-fold of non-fused GH. The invention further provides novel interferon and other cytokine IgG fusion proteins.

In the course of this work the inventors discovered that certain GH-, EPO- and G-CSF-IgG fusion proteins were secreted from mammalian cells as mixtures of monomers and disulfide-linked dimers. Previous studies had reported secretion of only disulfide-linked, dimeric IgG fusion proteins. For use as human therapeutics it will be desirable to use homogeneous populations of either monomeric or dimeric fusion proteins, but not mixtures of the two forms.

The inventors disclose methods for purifying monomeric and dimeric forms of the fusion proteins.

The inventors also discovered that purified GH-, EPO- and G-CSF-IgG-C_H fusion proteins had reduced specific activities in *in vitro* biological assays compared to the non-fused proteins and IgG-Fc fusion proteins. This invention discloses methods to improve the specific biological activities of IgG-C_H fusion proteins.

The invention also discloses EPO-, G-CSF- and GH-IgG fusion proteins with certain specific activities in *in vitro* biological assays but which possess reduced ability to activate complement and bind Fc receptors.

The invention also discloses methods for constructing covalent, multimeric cytokine fusion proteins that retain biological activity.

The teachings of this application are meant to include variants of the disclosed proteins that possess some or all of the biological properties of the disclosed proteins. In some cases the variants may possess additional properties, e.g., improved stability or bioactivity, not shared by the original protein. For example, Mark et al. (1984) disclose an IFN-β mutein in which cysteine at position 17 is replaced by serine. IFN-β (Ser-17) displays improved stability relative to wild type IFN-β. Lu et al. (1992) disclose a G-CSF mutein in which cysteine ay position 17 is replaced by serine. Kuga et al. (1989), Hanazono et al. (1990) and Okabe et al. (1990) describe additional G-CSF muteins with enhanced biological properties. Elliot and Byrne (1995) have described mutants of EPO with enhanced biological activities.

The IgG fusion proteins described herein can be used to treat the same human diseases as the non-fused proteins. For example, the EPO-IgG fusion proteins can be used to treat anemia resulting from kidney failure, chemotherapy and drug complications. The EPO-IgG fusion

proteins also can be used to stimulate red blood cell formation in normal individuals who wish to enhance their blood volume prior to surgery. The G-CSF-IgG fusion proteins can be used to treat neutropenia resulting from chemotherapy and drug complications, for mobilization of progenitor cells for collection in peripheral blood progenitor cell transplants and for treatment of severe chronic neutropenia. The GH-IgG fusion proteins can be used to treat short stature and cachexia. The IFN-α-IgG fusion proteins can be used to treat viral diseases and cancer. The IFN-β-IgG fusion protein can be used to treat multiple sclerosis, viral diseases and cancer. The IgG fusion proteins described herein will possess longer circulating half-lives in patients, which will allow the fusion proteins to be administered less frequently or in effective lower doses than the non-fused proteins. Typically, GH and G-CSF are administered by daily injections and EPO is administered by thrice weekly injections. The IgG fusion proteins described herein also will be useful as diagnostic reagents for identifying cells expressing receptors for EPO, G-CSF and GH. The IgG fusion proteins also will be useful as *in vitro* reagents for studying cell proliferation and differentiation.

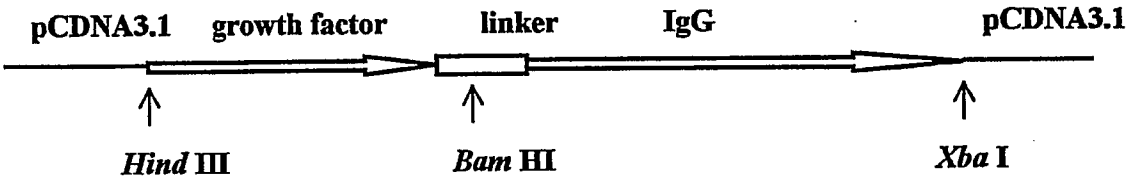
Example 1

Construction of Growth Factor-IgG Gene Fusions.

A. General Strategy.

Growth factor (GF)–IgG gene fusions were constructed as described below. The general strategy employed for these constructions is outlined here and the specifics of individual cloning steps are detailed below. Cloning of the IgG4-C_H coding sequence involved additional variations to the general strategy and these variations are described below. The human growth factor genes (GH, EPO and G-CSF) were cloned as cDNAs from various RNA sources detailed

below. PCR primers used in these clonings added an optimized Kozak sequence (GCCACCC; Kozak, 1991) and a *Hind* III restriction site to the 5' end of each these clones and a portion of a peptide linker (ser-gly-gly-ser) terminating in a *Bam* HI restriction site, to the 3' end of each of these clones. The growth factor genes were cloned as *Hind* III – *Bam* HI fragments into the mammalian cell expression vector pCDNA3.1(+) (Invitrogen, Inc.) and sequenced. In parallel, IgG coding sequences (IgG1-Fc, IgG1-C_H, IgG4-Fc, IgG4-C_H) were cloned from cDNAs generated from human leukocyte RNA. PCR forward primers used in these clonings incorporated a portion of a peptide linker (gly-ser-gly-gly-ser) containing a *Bam* HI restriction site at the 5' end of each of these clones. The reverse PCR primers were designed to anneal to the 3' untranslated regions of the IgG1 and IgG4 mRNAs (about 40 bp downstream of the translational stop codon) and included an *Xba* I restriction site. The IgG coding sequences were cloned into pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and confirmed by DNA sequencing. The fusion genes were then constructed by excising the IgG coding sequences as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::GF recombinant plasmids that had been cut with *Bam* HI and *Xba* I. In the resulting pCDNA3.1 constructs the fusion genes are transcribed by the strong cytomeglovirus immediate early promoter present in pCDNA3.1(+) upstream of the cloned fusion gene. Ligation of the two fragments through the *Bam* HI site within the linker sequence results in a seven amino acid linker (ser-gly-gly-ser-gly-gly-ser) at the fusion junction.



B. Cloning of Growth Factor Genes

1. Cloning of human Growth Hormone : A cDNA encoding human Growth Hormone (GH) was amplified from human pituitary single-stranded cDNA (CLONTECH, Inc., Palo Alto, CA), using the polymerase chain reaction (PCR) technique and primers BB87 (5> GCAAGCTTGCCACCATGGCTACAGGCTCCCGGACG >3) and BB88 (5> CGCGGATCCTCCGGAGAA GCCACAGCTGCCCTCCAC >3). Primer BB87 anneals to the 5' end of the coding sequence for the hGH secretion signal, whereas the reverse primer, BB88, anneals to the 3' end of the GH coding sequence. The resulting ~ 680 bp PCR product was digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector (Invitrogen, Inc., Carlsbad, CA) that had been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Martial et al., 1979; Roskam and Rougeon, 1979; Seeburg, 1982; DeNoto et al., 1981) was designated pCDNA3.1(+)::GHfus or pBBT159.

2. Cloning of human Erythropoietin . A cDNA encoding human erythropoietin (EPO) was cloned by PCR using forward primer BB45 (5> CCCGGAT CCATGGGGGTGCACGAATGTCCTG >3) and reverse primer BB47 (5> CCCGAATTCTATGCCCAGGT GGACACACCTG >3). BB45 anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the EPO signal sequence and contains a *Bam* HI site for cloning purposes. BB47 anneals to the 3' untranslated region of the EPO mRNA immediately downstream of the translational stop signal and contains an *Eco* RI restriction site for cloning purposes. Total RNA isolated from the human liver cell line Hep3B was used in first strand synthesis of single-stranded cDNA for PCR. For preparation of total cellular RNA, Hep3B cells (available from the American Type Culture Collection, Rockville,

MD) were grown in Delbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). EPO expression was induced by treating the cells for 18 h with 130 μM Deferoxamine or 100 μM cobalt chloride. Both compounds have been shown to induce EPO mRNA and protein expression in Hep 3B cells (Wang and Semenza, 1993). RNA was isolated from the cells using an RNeasy Mini kit (Qiagen), following the manufacturer's directions. Approximately 320 μg of total RNA was isolated from 1.4 $x10^7$ cells treated with cobalt chloride and 270 μg of total RNA isolated from 1.4 $x10^7$ cells treated with Deferoxamine.

First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corporation (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand syntheses as templates were carried out with primers BB45 and BB47. The expected ~ 600 bp PCR product was observed when reaction products were run out on an agarose gel. Both RNA preparations yielded an EPO PCR product. The PCR product was digested with *Bam* HI and *Eco* RI and cloned into vector pUC19 that had been cut with *Bam* HI and *Eco* RI and treated with alkaline phosphatase. DNA sequencing identified a clone containing the correct coding sequence for the EPO gene. This plasmid was designated pBBT131.

Plasmid pBBT131was used as template in a PCR reaction with primers BB89 (5>CGCAAGCTTGCCACCATGGGGGTGC ACGAATGTCCT >3) and BB90 (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTGCAGGC >3) to construct a modified EPO cDNA suitable for fusion with IgG genes. Primer BB89 anneals to the 5' end of the coding sequence for the EPO secretion signal and the reverse primer, BB90, anneals to the 3' end of the EPO coding sequence. The resulting ~ 610 bp PCR product was digested with *Hind* III and *Bam*

HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Lin et al., 1985) was designated pCDNA3.1(+)::EPOfus or pBBT176.

Similar procedures can be used to create modified EPO cDNAs in which Arg166 is deleted. In this case a primer with the sequence (5>CGCGGATCCTCCGGATCTGTCCCCTGTCCTG CAGGC >3) should be used in place of primer BB90.

3. Cloning of human Granulocyte Colony-Stimulating Factor. A cDNA encoding human Granulocyte Colony Stimulating Factor (G-CSF) was amplified by PCR from total RNA isolated from the human bladder carcinoma cell line 5637 (available from the American Type Culture Collection, Rockville, MD). The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita, CA) following the manufacturer's directions. Approximately 560 μg of total RNA was isolated from 4.5 $x10^7$ cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of the first strand synthesis as template were carried out with forward primer BB91 (5>CGCAAGCTTGCCACCATGGCTGGACC TGCCACCCAG>3 and reverse primer BB92 (5>CGCGGATCCTCCGGAGGGCTGGGCAAGGTGGCGTAG >3). Primer BB91 anneals to the 5' end of the coding sequence for the G-CSF secretion signal and the reverse primer, BB92, anneals to the 3' end of the G-CSF coding sequence. The resulting ~ 640

bp PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Souza et al., 1986; Nagata et al., 1986a,b) was designated pCDNA3.1(+)::G-CSFfus or pBBT165.

C. Cloning of IgG coding sequences.

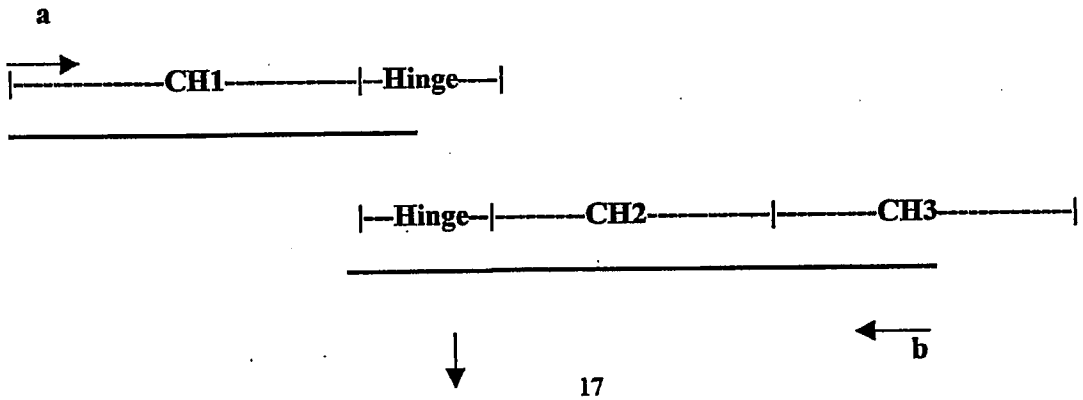
1. Cloning of IgG1-Fc coding sequences. A cDNA encoding IgG1-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH, Inc., Palo alto, CA) by PCR using primers BB83 (5>CGCGGATCCG GTGGCTCAGAGCCCAAATCTTGTGACAAAACT >3) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB83 anneals to the 5' end of the coding sequence of the hinge domain of IgG1, whereas the reverse primer BB82 anneals to the 3' untranslated region of IgG1 and IgG4 mRNA ~ 45 bp downstream of the translational stop codon. The IgG1 and IgG4 sequences are identical over the 21 bp segment to which BB82 anneals. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Bam* HI and *Xba* I, alkaline phosphtase treated, and gel purified. Two clones were sequenced but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique restriction sites (*Sac* II in the CH2 domain of IgG1 and *Bgl* II in the pCDNA3.1(+) vector) to construct a full length IgG1-Fc clone in pCDNA3.1(+) via *in vitro* recombination. The resulting clone, which had the correct IgG1-Fc sequence, was designated pCDNA3.1(+)::fusIgG1-Fc or pBBT167.

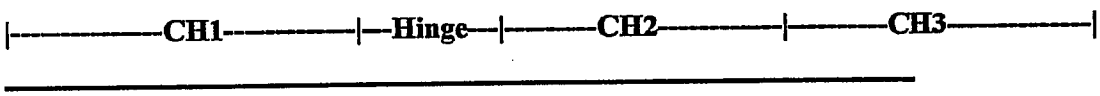
2. Cloning of IgG4-Fc coding sequences. A cDNA encoding IgG4-Fc (hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using primers BB84 (5>CGCGGATCCGG TGGCTCAGAGTCCAAATATGGTCCCCCATGC >3) and BB82 (5>CGCTCTAG AGGTACGTGCCAAGCA TCCTCG>3). Forward primer BB84 anneals to the 5' end of the coding sequence of the hinge domain of IgG4. The reverse primer BB82 is described above. The ~ 790 bp PCR product was digested with *Bam* HI and *Xba* I and cloned into pCDNA3.1(+) that had been similarly digested. A clone with the correct DNA sequence (Ellison et al., 1981) was designated pCDNA3.1(+)::fusIgG4-Fc or pBBT158.

3. Cloning of IgG1-C_H coding sequences. A cDNA encoding IgG1-C_H (CH1-hinge-CH2-CH3 domains) was amplified from human leukocyte single-stranded cDNA (CLONTECH) by PCR using BB81 (5>CGCGGATCC GGTGGCTCAGCCTCCACCAAGGGCCCATC>3) and BB82 (5>CGCTCTAGAGGTACGTGCCAAGC ATCCTCG>3). Forward primer BB81 anneals to the 5' end of the coding sequence of the CH1 domain of IgG1 and IgG4. The sequences at the 5' end of the CH1 domains of these two exons are almost identical: 19/20 nucleotides match. The reverse primer, BB82, is described above. The ~ 1080 bp PCR product was digested with *Bam* HI and *Xba* I, gel purified and cloned into pCDNA3.1(+) that had been digested similarly. These primers in principle could amplify both IgG1 and IgG4 sequences. Since IgG1 is much more abundant in serum than IgG4 (Roitt et al., 1989) we expected that most clones would encode IgG1. The first two clones sequenced were IgG1 but each contained a single base pair substitution that resulted in an amino acid substitution mutation. Otherwise the sequences obtained matched the published human IgG1 genomic DNA sequence (Ellison et al., 1982). The relative positions of the mutations in the two clones allowed us to use convenient unique

restriction sites (*Age* I in the CH1 domain of IgG1 and *Bst* BI in the pCDNA3.1(+) vector) to construct a full length IgG1-C_H clone in pCDNA3.1(+) via *in vitro* recombination. A clone with the correct IgG1-C_H sequence was designated pCDNA3.1(+)::fusIgG1-C_H or pBBT166.

4. Cloning of IgG4-C_H coding sequences. The near identity of the DNA sequences encoding the 5' ends of the IgG1 and IgG4 CH1 domains and the relatively low abundance of the IgG4 mRNA led us to an alternative strategy for cloning the IgG4-C_H coding sequences. We used PCR-based site directed mutagenesis to change the DNA sequence of the cloned IgG1 CH1 domain to match the amino acid sequence of the IgG4 CH1 domain. The CH1 domains differ at only 8 of 98 nucleotides and these positions are clustered, so that one round of PCR using two mutagenic oligos can convert the IgG1 CH1 sequence into the IgG4 CH1 sequence. A second round of PCR added the *Bam* HI site and linker sequence to the 5' end of the IgG4 CH1 and 21 bp of sequence from the IgG4 Hinge domain to the 3' end. The technique of "gene splicing by overlap extension" (Horton et al., 1993) was then employed to recombine the engineered IgG4 CH1 domain with the IgG4 Fc (Hinge-CH2-CH3) sequence. In this technique two separate fragments sharing a segment of identical sequence, the "overlap", at one end are extended through the annealed overlap regions in a PCR reaction as diagrammed below.





To construct the IgG4 CH1 sequence, mutagenic primers BB119 (5> TCCACCAAG GGCCCATCCGT CTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGC ACAGC>3) and BB120 (5> TCTCTTG TCCACCTTGGTGTTGCTGGGCTTGTGATC TACGTTGCAGGTGTAGGTCTTCGTGCCCAA >3) were used in PCR reactions with pBBT166, which carries the cloned IgG1-C_H sequence as described above. Forward primer BB119 anneals to the sequence encoding amino acids 2 through 23 of the CH1domain and encodes 4 amino acid substitutions: S14C, K16R, G20E and G21S. Reverse primer BB120 anneals to the sequence encoding amino acids 76 through 97 of the CH1 domain and encodes 4 additional amino acid substitutions: Q79K, I82T, N86D and K97R. The ~ 290 bp product of this PCR reaction was gel purified and used template in a PCR reaction with primers BB81(see above) and BB121(5>TGGGGGACCATATTTG GACTCAACTCTCTTGTCCACCTT >3). Reverse primer BB121 anneals to the 3' end of the CH1 domain of IgG4, adds amino acid 98 of the CH1 domain and 21 bp extending into the Hinge domain of IgG4. The ~ 330 bp product of this reaction was gel purified and used as one of the template molecules in the PCR splicing reaction. The other template for the splicing reaction was generated by PCR of the cloned IgG4-Fc sequence of pBBT158 (described above) with primers BB84 and BB82 which amplify the IgG4 Fc domain as described above. The resulting ~ 790 bp product consists of the IgG4 hinge-CH2-CH3 sequence. This fragment was gel purified and used as one of the template molecules in the PCR splicing reaction. This reaction employed the primers BB81 and BB82 and generated a full-length "spliced" product of ~ 1075 bp. To minimize the DNA sequencing required to

confirm this product, the PCR fragment was digested with *Bam* HI and *Sac* II and the ~ 530 bp fragment (containing the complete CH1 and hinge domains and a portion of the CH2 domain) was cloned into pBC-SK+ (Stratagene) for sequencing. The sequence of the *Bam* HI – *Sac* II fragment was confirmed for one clone which was the designated pBBT182. The *Bam* HI – *Sac* II fragment of pBBT182 was then used convert the GF–IgG4-Fc clones to full length GF–IgG4-C_H clones as detailed below.

D. Construction of Growth Factor–IgG (GF-IgG) fusions.

Most (9/12) of the growth factor-IgG gene fusions were generated by excising the IgG coding sequences cloned in pCDNA3.1(+) as *Bam* HI – *Xba* I fragments and cloning these fragments into the pCDNA::[GF] recombinant plasmids which had been cut with *Bam* HI and *Xba* I. The fusions of the three growth factor genes to IgG4-C_H were constructed by excising the ~ 530 bp *Bam* HI– *Sac* II fragment of pBBT182 and replacing the ~ 240 bp *Bam* HI- *Sac* II fragments of the three pCDNA::[GF]-IgG4-Fc clones. The resulting plasmids and the GF-IgG fusion proteins they encode are listed in Table 1.

Example 2

Expression and Purification of GF-IgG Fusion Proteins

A. Small Scale Transfection of COS Cells

Expression and bioactivity of the GF-IgG fusion proteins were assessed initially by small-scale transfection of COS cells. Endotoxin-free plasmid DNAs were prepared using an "Endo-Free Plasmid Purification Kit" (Qiagen, Inc.) according to the vendor protocol and used to transfect COS-1 cells (available from the American Type Culture Collection, Rockville, MD). The COS-1 cells were Delbecco's Modified Eagle's Media supplemented with 10% FBS,

50units/ml penicillin, 50μg/ml streptomycin and 2mM glutamine (growth media). Initial transfection experiments were carried out in Costar 6 well tissue culture plates (VWR Scientific) using the following protocol. Briefly, 2-3 x 10^5 cells were seeded into each well in 2 ml of growth media and allowed to incubate overnight at 37°C and 5% CO_2 by which time the cells had reached 50-60% confluency. For each well, 0.8 μg of plasmid DNA was complexed with 6 μl of LipofectAMINE reagent (Gibco BRL, Gaithersburg, MD) in 186 μl of OPTI-MEM I Reduced Serum Medium (Gibco BRL, Gaithersburg, MD) for 30-45 minutes at room temperature. COS-1 cells were washed one time with 2ml of OPTI-MEM I per well and then 1.8 ml of OPTI-MEM I was added to each well. The complex mixture was then added to the well and left at 37°C, 5% CO_2 for approximately 4-5 hours. After the incubation period, the mixture was replaced with 2 ml of growth media per well and left overnight at 37°C, 5% CO_2. The next day the cells were washed twice with 2ml of DMEM (no additives) per well. Following the wash steps, 2 ml of serum-free growth media was added to each well and the cells left at 37°C, 5% CO_2. Conditioned media containing the GF-IgG-fusion proteins were harvested after 72 hours and analyzed by SDS-PAGE and Western blot to confirm expression of the GF-IgG-fusion proteins. The parent plasmid, pCDNA 3.1(+) (Invitrogen) was used as a negative control. Transfection efficiency was estimated to be ~15%, using pCMVβ (Clontech), which expresses *E. coli* β-galactosidase. Transfected cells expressing β-galactosidase were identified using a β-Gal Staining Set (Boehringer Mannheim, Indianapolis, IN).

Samples of the conditioned media were prepared in SDS-PAGE sample buffer with the addition of 1% β-mercaptoethanol (BME) when desirable and electrophoresed on precast 14% Tris-glycine polyacrylamide gels (Novex). Western blots using appropriate antisera demonstrated expression of all of the GF-IgG fusion proteins (data not shown – see purified

proteins below). The GH-IgG fusion proteins were detected using a polyclonal rabbit anti-synthetic-hGH antiserum (kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary Program). The EPO- and G-CSF-IgG fusion proteins were detected using polyclonal antisera purchased from R&D Systems (Minneapolis, MN). Serial dilutions of the conditioned media were analyzed in the appropriate *in vitro* bioassays described later. These assays demonstrated significant activity in the conditioned media and encouraged us to perform large-scale transfections so that the proteins could be purified for specific activity measurements.

B. Large Scale Transfection of COS-1 Cells

Large scale transfections were carried out using Corning 100 mm tissue culture dishes or Corning T-75 tissue culture flasks (VWR Scientific). For 100 mm dishes, 1.6×10^6 cells were plated in 10 ml of growth media per dish and incubated at 37°C, 5% CO_2 overnight. For each 100 mm dish, 6.7 µg endotoxin-free plasmid DNA was complexed with 50 µl of LipofecAMINE reagent in 1.5 ml of OPTI-MEM I for 30-45minutes at room temperature. The COS-1 cells were washed one time with 10 ml OPTI-MEM per dish and then replaced with 6.6 ml of OPTI-MEM I. Following complex formation, 1.67 ml of the complex was added to each dish and left at 37°C, 5% CO_2 for 4-5 hours. After the incubation period, the reaction mixture was replaced with 10 ml of serum containing growth media per dish and left at 37°C, 5% CO_2 overnight. The next day the cells were washed twice with 10 ml of DMEM (no additives) per dish. Following the wash steps, 10ml of serum-free growth media was added to each dish and incubated at 37°C, 5% CO_2. Conditioned media were harvested routinely every three days (on days 3, 6, 9 and 12) and fresh serum-free growth media added to the cells. Transfections in T-75 culture flasks were identical to the 100mm dish protocol with the following exceptions: Cells were plated at 2×10^6

cells per flask and 9.35 μg of endotoxin-free plasmid DNA was complexed with 70 μl of LipofectAMINE reagent in 2.1ml of OPTI-MEM I for each T-75 flask. Following complex formation, 2.3 ml of the complex was added to each flask containing 7.7 ml of OPTI-MEM I. Transfection efficiency was determined to be ~15% using pCMVβ and staining for β-galactosidase expression as decribed earlier. The 12 plasmids listed in Table 1 were transfected into COS-1 cells using the large-scale format to generate protein for purification. The conditioned media were clarified by centrifugation and stored at -20°C for later purification. Western blots were used to confirm expression of the IgG-fusion proteins.

C. Purification of GF-IgG-Fusion Proteins

Approximately 300 ml of transfected COS-1 cell conditioned media for each IgG-fusion protein was pooled and concentrated using an Ultrafiltration cell and either a YM3 or YM30 DIAFLO Ultrafiltration membrane (Amicon, Beverly, MA). Concentrated pools were then loaded onto a 1ml Pharmacia HiTrap recombinant Protein A column previously equilibrated with 20 mM NaPhosphate pH 7.0. The column was washed with 20 mM NaPhosphate until the A_{280} had reached baseline. Bound protein was eluted with 100 mM NaCitrate pH 3.0 and collected into sufficient 1M Tris pH 9.0 to achieve a final pH of approximately 7.0. Each fusion protein was purified using a dedicated column to avoid any possibility of cross-contamination. All of the IgG fusion proteins chromatographed similarly, yielding a single peak in the elution step. Column fractions were analyzed using 8-16% precast Tris-glycine SDS-PAGE and fractions enriched for the IgG-fusion protein were pooled. Protein concentrations of the pooled fractions were determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum

albumin (BSA) as the standard. Recoveries of the various purified GF-IgG fusion proteins are given in Table 1 and ranged from 96 to 376 μg per 300 ml of conditioned media.

Figure 1 displays SDS gels of the purified proteins under reducing and non-reducing conditions and stained with Coomassie blue. All of the GF-IgG fusion proteins were recovered principally as disulfide-linked dimers. The molecular weights of the proteins ranged from 115-190 kDa kDa under non-reducing conditions and 50-70 kDa under reducing conditions, largely consistent with the molecular weights predicted in Table 1. The molecular weights of the EPO-IgG fusion proteins were the only ones larger than predicted (see Figure 1), presumably due to extensive glycosylation of the EPO domain. Monomeric fusion proteins were present in all of the samples, but were more abundant with the IgG4 fusion proteins (they can be seen in the non-reduced gels in Figure 1). The sizes of the major IgG fusion protein bands were different from the molecular weights of bovine IgG (see Figure 1), indicating that the proteins purified were not contaminating bovine IgGs from serum used in the experiments. The major IgG fusion protein bands also reacted with antisera specific for GH, EPO and G-CSF in Western blots of the samples. Purity of the IgG fusion proteins was estimated to be at least 90% from Coomassie blue staining of the gels.

All of the GF-IgG-CH fusions contained a large aggregate that migrated at the top of the gel when the samples were analyzed under non-reducing conditions. This aggregate disappeared when the samples were analyzed under reducing conditions and the amount of protein at the molecular weight of the major GF-IgG-C_H bands seemed to increase proportionately. The aggregates also reacted with antisera specific for the various growth factors. These data suggest the aggregates are disulfide-linked multimers of the GF-IgG-C_H fusion proteins. Under reducing SDS-PAGE conditions, all of the GF-IgG-C_H fusions show a diffuse band approximately 20 kDa

larger than the main GF-IgG-C_H band. This band reacted with antisera against the growth factors and may be related to the aggregates.

Table 1 Predicted Molecular Weights and Recoveries of GF-IgG Fusion Proteins

Expression Plasmid	IgG-Fusion Protein	Predicted Molecular Weight (kDa)[1]		Protein Recovery µg / 300 ml
		Monomer	Dimer	
PBBT 171	GH-IgG1-C_H	58,706	117,412	376
PBBT 172	GH-IgG1-Fc	48,693	97,386	248
PBBT183	GH-IgG4-C_H	58,541	117,082	ND[2]
PBBT 163	GH-IgG4-Fc	48,365	96,730	96
PBBT 173	G-CSF-IgG1-C_H	55,564	111,128	122
PBBT 174	G-CSF-IgG1-Fc	45,551	91,102	122
PBBT 184	G-CSF-IgG4-C_H	55,399	110,798	ND
PBBT 175	G-CSF-IgG4-Fc	45,222	90,444	96
PBBT 179	EPO-IgG1-C_H	54,972	109,944	133
PBBT 180	EPO-IgG1-Fc	44,960	89,920	235
PBBT 185	EPO-IgG4-C_H	54,808	109,616	ND
PBBT 181	EPO-IgG4-Fc	44,632	89,264	257

[1] Does not include molecular weight contributions due to of glycosylation.

[2] Not determined

Example 3

In Vitro Bioactivities of Purified IgG Fusion Proteins

A. General Strategy

Cell proliferation assays were developed to measure *in vitro* bioactivities of the IgG fusion proteins. The assays measure uptake and bioreduction of the tetrazolium salt MTS [3-(4,5-dimethylthiazol-2-yl)-5-3-carboxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium]. In the presence of

an electron coupler such as phenazine methosulfate (PMS), MTS is converted to a formazan product that is soluble in tissue culture media and can be measured directly at 490 nm. Cell number is linear with absorbance values up to about 2. For EPO and G-CSF we were able to use existing cell lines to develop the bioassays. For GH, we needed to create a cell line that proliferates in response to GH. Such a cell line was created by stably transforming a murine leukemia cell line with a rabbit GH receptor.

In general, the bioassays were set up by washing the appropriate cells three times with media (no additives) and resuspending the cells at a concentration of $0.7\text{-}1x10^5$/ml in media with additives (media used for each cell line is given below). Fifty µl ($3.5\text{-}5x10^3$ cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial dilutions of the protein samples to be tested were prepared in serum-containing media. Fifty µl of the diluted protein samples were added to the test wells and the plates incubated at 37^0C in a humidified 5% CO_2 tissue culture incubator. Protein samples were assayed in triplicate wells. After 60-72 h, 20 µl of an MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) was added to each well and the plates incubate at 37^0C in the tissue culture incubator for 1-4 h. Absorbance of the wells was read at 490 nm using a microplate reader. Control wells contained media but no cells. Mean absorbance values for the triplicate control wells were subtracted from mean values obtained for the test wells. EC_{50}s, the amount of protein required for half maximal stimulation, was calculated for each sample and used to compare bioactivities of the proteins. Non-glycosylated molecular weights were used in the molar ratio calculations for consistency. Non-glycosylated molecular weights of 18,936, 18,987 and 22,129 were assumed for EPO, G-CSF and GH, respectively. Monomer molecular weights were used in the calculations for the IgG fusion proteins. Using molecular weights of the

monomer fusion proteins estimated from SDS gels (50-70 kDa) and glycosylated molecular weights of 35kDa for EPO and 19.7 kDa for G-CSF (GH is not glycosylated) gave similar activity ratios.

B. Bioactivities of EPO-IgG Fusion Proteins

The human UT7/epo cell line was obtained from Dr. F. Bunn of Harvard Medical School, Boston, MA. This cell line proliferates in response to EPO and is dependent upon EPO for cell survival (Boissel et al., 1993). The cells were maintained in Iscove's Modified Delbecco's Media (IMDM) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 1 unit/ml recombinant human EPO (CHO cell-expressed; R&D Systems). Bioassays were performed in cell maintenance media using the procedures described above. Serial dilutions of recombinant CHO cell-expressed human rEPO (R&D Systems) were analyzed in parallel.

The UT7/epo cell line shows a strong proliferative response to rEPO, as evidenced by a dose-dependent increase in cell number and absorbance values. In the absence of rEPO, the majority of UT7/epo cells die, giving absorbance values less than 0.1. Commercial CHO cell-expressed rEPO had a mean EC_{50} of approximately 0.6 ng/ml in the bioassay (Table 2). This value agrees with EC_{50} values reported in the R&D Systems specifications (0.05 – 0.1 unit/ml or approximately 0.4-0.8 ng/ml). The EPO-IgG1-Fc and IgG4-Fc fusion proteins had identical EC50's of approximately 1.3 ng/ml in the bioassay (Table 2). On a molar basis, the EC_{50}s of CHO-cell expressed rEPO and the EPO-IgG-Fc fusions were identical (approximately 30 pM; Table 2). The EPO-IgG1-C_H fusion protein had an EC_{50} of 3.1 ng/ml or 60 pM (Table 2), which represents an approximate 2-fold reduction in specific activity relative to the EPO-IgG-Fc fusion proteins and non-fused rEPO. The EPO-IgG4-C_H fusion protein had a mean EC_{50} of 2.05 ng/ml.

Table 2. Bioactivities of EPO-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (ng/ml)[1]	Mean EC_{50}	
			ng/ml	pM
-	RhEPO (CHO)	0.52, 0.55, 0.60	0.56	30
pBBT180	EPO-IgG1-Fc	1.1, 1.2, 1.5	1.27	28
pBBT181	EPO-IgG4-Fc	1.1, 1.2, 1.5	1.27	29
pBBT179	EPO-IgG1-C_H	2.9, 3.0, 3.5	3.13	57
pBBT185	EPO-IgG4-C_H	2.0, 2.1	2.05	37

[1] Data from individual experiments

C. Bioactivities of G-CSF-IgG Fusion Proteins

The murine NFS60 cell line was obtained from Dr. J. Ihle of the University of Tennessee Medical School, Memphis Tennessee. This cell line proliferates in response to human or mouse G-CSF or IL-3 (Weinstein et al., 1986). The cells were maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin, 50 µg/ml streptomycin and 17-170 units/ml mouse IL-3 (R&D Systems). Assays were performed in cell maintenance media using the procedures described above. Serial dilutions of recombinant human G-CSF (*E. coli*-expressed; R&D Systems) were analyzed in parallel.

The NFS60 cell line shows a strong proliferative response to rhG-CSF, as evidenced by a dose-dependent increase in cell number and absorbance values. rhG-CSF had a mean EC_{50} of 18 pg/ml in the bioassay (Table 3). This value agrees with the EC_{50} value reported in the R&D Systems specifications (10-30 pg/ml). The G-CSF-IgG1-Fc and G-CSF-IgG4-Fc fusion proteins had mean EC_{50}'s of 34 and 50 pg/ml, respectively, in the bioassay (Table 3). On a molar basis, the EC_{50}s of rhG-CSF and the G-CSF-IgG1-Fc fusions were similar (approximately 0.9 pM; Table 3), whereas the EC_{50} of the G-CSF-IgG4-Fc fusion protein was reduced slightly

(1.25 pM). The G-CSF-IgG1-C_H fusion protein had a mean EC_{50} of 182 pg/ml or 3.2 pM (Table 3), which represents an approximate 3-fold reduction in specific activity relative to G-CSF-IgG1-Fc fusion protein and non-fused rhG-CSF.

Table 3. Bioactivities of G-CSF-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (pg/ml) [1]	Mean EC_{50}	
			pg/ml	pM
-	rhG-CSF	17, 18, 18	17.7	0.93
PBBT174	G-CSF-IgG1-Fc	34, 39, 42	38.3	0.84
PBBT175	G-CSF-IgG4-Fc	50, 59, 61	56.7	1.25
PBBT173	G-CSF-IgG1-C_H	160, 190, 195	182	3.2
PBBT184	G-CSF-IgG4-C_H	ND [2]	-	-

[1] Data from individual experiments

[2] Not determined

D. Bioactivities of GH-IgG Fusion Proteins:

1. Development of an *in vitro* bioassay for hGH: We created a cell line that proliferates in response to GH by stably transforming a murine leukemis cell line, FDC-P1 with a plasmid that directs expression of a rabbit GH receptor. We cloned the rabbit GH receptor via RT-PCR from polyA+ rabbit liver RNA (CLONTECH) into pCDNA3.1(+). The resulting plasmid, termed pBBT118, was used to construct a stably tranformed FDC-P1 cell line that expresses the receptor. hGH binds human and rabbit GH receptors with similar affinities (Leung et al, 1987; Hammonds et al , 1991; Rowlinson et al., 1995). Rowlinson et al. (1995) described an hGH cell proliferation assay that uses the FDC-P1 cell line stably tranfected with the rabbit GH receptor.

2. Cloning a cDNA Encoding the Rabbit GH Receptor: The rabbit GH receptor was cloned by PCR using forward primer BB3 (5'-CCCCGGATCCGCCACCATGGATCTCTGG CAGCTGCTGTT-3') and reverse primer BB36 (5'- CCCCGTCGACTCTAGAGCCATTA GATACAAAGCTCT TGGG-3'). BB3 anneals to the DNA sequence encoding the initiator methionine and amino terminal portion of the receptor. BB3 contains an optimized KOZAK sequence preceding the initiator methionine and a *Bam* HI site for cloning purposes. BB36 anneals to the 3' untranslated region of the rabbit GH receptor mRNA and contains *Xba* I and *Sal* I restriction sites for cloning purposes. Rabbit liver poly(A)$^+$ mRNA (purchased from CLONTECH, Inc.) was used as the substrate in first strand synthesis of single-stranded cDNA to produce template for PCR amplification. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp. Parallel first strand cDNA syntheses were performed using random hexamers or BB36 as the primer. Subsequent PCR reactions using the products of the first strand syntheses as templates were carried out with primers BB3 and BB36. The expected ~ 1.9 kb PCR product was observed in PCR reactions using random hexamer-primed or BB36-primed cDNA as template. The random hexamer-primed cDNA was digested with *Bam* HI and *Xba* I, which generates two fragments (~ 365 bp and ~ 1600 bp) because the rabbit GH receptor gene contains an internal *Bam* HI site. Both fragments were gel-purified. The full-length rabbit GH receptor cDNA was then cloned in two steps. First the ~1600 bp *Bam* HI - *Xba* I fragment was cloned into pCDNA3.1(+) hat had been digested with these same two enzymes. These clones were readily obtained at reasonable frequencies and showed no evidence of deletions as determined by restriction digests and subsequent sequencing. To complete the rabbit receptor cDNA clone, one of the sequenced plasmids containing the 1600 bp *Bam* HI - *Xba* I fragment was digested with *Bam* HI, treated with Calf Alkaline Phosphatase, gel-purified and ligated with

the gel purified ~365 bp *Bam* HI fragment that contains the 5' portion of the rabbit GH receptor gene. Transformants from this ligation were picked and analyzed by restriction digestion and PCR to confirm the presence of the ~365 bp fragment and to determine its orientation relative to the distal segment of the rabbit GH receptor gene. The sequence for one full length clone was then verified. This plasmid, designated pBBT118, was used to stably transfect FDC-P1 cells.

3. Selection of Stably Transfected FDC-P1 Cells Expressing the Rabbit GH Receptor: Endotoxin-free pBBT118 DNA was prepared using a Qiagen "Endo-Free Plasmid Purification Kit" and used to tranfect FDC-P1 cells. The mouse FDC-P1 cell line was purchased from the American Type Culture Collection and routinely propagated in RPMI 1640 media supplemented with 10% fetal calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 17-170 Units/ml mouse IL-3 (FDC-P1 media). Mouse IL-3 was purchased from R&D Systems. FDC-P1 cells were transfected with plasmid pBBT118 using DMRIE-C cationized lipid reagent purchased from GIBCO, following the manufacturer's recommended directions. The next day transfected cells were transferred to T-75 tissue culture flasks containing 15 ml FDC-P1 media supplemented with IL-3 (17U/ml), hGH (5nM) and 10% horse serum rather than fetal calf serum. Horse serum was used because of reports that fetal calf serum contains a growth-promoting activity for FDC-P1 cells. Three days later the cells were centrifuged and resuspended in fresh FDC-P1 media containing 400 ug/ml G418, 17U/ml IL-3, 5nM hGH, 10% horse serum and incubated at 37^0C. Media was changed every few days. The cells from each transfection were split into T-75 tissue culture flasks containing fresh media and either mouse IL-3 (17 U/ml) or hGH (5nM). G418 resistant cells were obtained from both the IL-3- and hGH-containing flasks. The transformants used in the bioassays originated from flasks containing hGH. Twelve independent cell lines were selected by limiting dilution. Five of the cell lines (GH-R3, -R4, -

R5, -R6 and -R9) showed a good proliferative response to hGH. Preliminary experiments indicated that the EC_{50} for hGH was similar for each cell line, although the magnitude of the growth response varied depending upon the line. The GH-R4 cell line was studied in most detail and was used for the assays presented below. The cell lines were routinely propagated in RPMI 1640 media containing 10% horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 400 μg/ml G418 and 2-5 nM pituitary hGH.

4. Bioassay results for Pituitary hGH and GH-IgG Fusion Proteins: Protein samples were assayed as described previously using RPMI media supplemented with 10% horse serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 400 μg/ml G418. All assays included a human pituitary GH standard. Experiments utilizing the parental FDC-P1 cell line were performed in the same way except that the assay media did not contain G418 and FBS was substituted for horse serum. The parental FDC-P1 cell line shows a strong proliferative response to mouse IL-3, but not to pituitary hGH. In the absence of IL-3, the majority of FDC-P1 cells die, giving absorbance values less than 0.2. In contrast, FDC-P1cells transformed with the rabbit growth hormone receptor proliferate in response to pituitary hGH, as evidenced by a dose-dependent increase in cell number and absorbance values. The EC_{50} for this effect ranged from 0.75-0.85 ng/ml pituitary hGH (0.03-0.04 nM) in different experiments, similar to what has been reported in the literature (Rowlinson et al., 1995). A significant difference between the parental FDC-P1 line and FDC-P1 cells transformed with the rabbit growth hormone receptor is that the latter cells survive in the absence of IL-3 or hGH, resulting in higher absorbance values (typically 0.6 – 1.1, depending upon the assay and length of incubation with MTS) in the zero growth factor control wells. The initial pool of rabbit growth hormone receptor transformants and all five independent

growth hormone receptor cell lines isolated showed the same effect. A similar result was obtained with a second set of independently isolated rabbit growth hormone receptor transfectants. Rowlinson et al. (1995) observed a similar effect, suggesting that IL-3/GH-independent survival is a consequence of the transformation procedure. Although the growth hormone receptor cell lines did not require IL-3 or hGH for growth, they still showed a robust proliferative response to IL-3 and hGH. The practical effect of the higher absorbance values in the absence of hGH is to decrease the "window" of the hGH response (the difference between the maximum and minimum absorbance values). This window consistently ranged from 30 - 100% of the zero growth factor values, similar to what was reported by Rowlinson et al. (1995).

All of the GH-IgG fusion proteins were active in the bioassay, but displayed reduced specific activities compared to pituitary hGH (Table 4). The GH-IgG1-Fc fusion protein was the most active, possessing an EC50 of 7-8 ng/ml (~0.16 nM). The GH-IgG4-Fc fusion protein was about two-fold less active than the IgG1 fusion protein, with a mean EC_{50} of 17 ng/ml (0.35 nM). The GH-IgG1-C_H fusion protein had the lowest activity, with an EC_{50} of 35 ng/ml (0.6nM). On a molar basis, bioactivities of the fusion proteins were reduced 4-fold (GH-IgG1-Fc), 10-fold (GH-IgG4-Fc) and 17-fold (GH-IgG1-C_H) relative to pituitary hGH.

Table 4. Bioactivities of GH-IgG Fusion Proteins

Clone	Protein	EC_{50} Range (ng/ml) [1]	Mean EC_{50}	
			ng/ml	nM
-	Pituitary hGH	0.75, 0.75, 0.85, 0.85	0.8	0.036
PBBT172	GH-IgG1-Fc	6.5, 7.0, 7.8, 9	7.6	0.160
PBBT163	GH-IgG4-Fc	15, 16, 18, 18	17	0.350
PBBT171	GH-IgG1-C_H	28, 32, 40, 40	35	0.600
PBBT183	GH-IgG4-C_H	ND [2]	-	-

[1] Data from individual experiments

[2] Not determined

Example 4

Eliminate or Minimize the Linker in the GF-IgG Fusion Proteins.

GF-IgG fusion proteins in which the 7 amino acid linker [ser-gly-gly-ser-gly-gly-ser] that fuses the GF to the IgG domain is eliminated or reduced to 2 to 4 amino acids can be constructed as described below. Similar methods can be used to create linkers shorter than 7 amino acids, to create linkers longer than 7 amino acids and to create linkers containing other amino acid sequences. The experiments described below use IgG1-Fc and EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA,IgD and IgE antibodies. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in **Examples 2 and 3.**

GF-IgG fusions without a linker can be created using PCR based "gene splicing by overlap extension" as described in **Example 1** (Horton et al., 1993). One can generate PCR products consisting of the IgG1-Fc coding sequence with a short 5' extension, consisting of the 3' terminal ~ 15 bp of coding sequence of EPO or G-CSF fused directly to the hinge coding

sequence. At the same time one can generate PCR products consisting of the EPO or G-CSF coding sequences with a short 3' extension consisting of the first 15 bp of the hinge coding sequence fused directly to the EPO or G-CSF coding sequence. The growth factor fragments and the IgG1-Fc fragments can then be spliced together via PCR "Sewing" (Horton et al., 1993) to generate direct fusions. These PCR products can be digested with appropriate restriction enzymes to generate relatively small DNA segments that span the fusion point and which can be readily cloned into similarly cut vectors pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc for sequence confirmation and COS cell expression. Cloning these smaller DNA fragments will minimize the sequencing that will need to be done to confirm the sequences of the direct fusions.

To construct a di-peptide [ser-gly] linker, one can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment can be digested with *Bsp* EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the linker [**ser-gly**-gly-ser-gly-gly-ser] so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker. The sequence of the newly inserted ~ 250 bp *Bsp* EI – *Sac* II fragment should be verified.

A similar procedure can be used to construct the 4 amino acid [ser-gly-gly-ser] linker. One can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCGGATCC to the hinge coding sequence. The GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly-ser. This PCR fragment can be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut

pCDN3.1(+)::EPO-IgG1-Fc and pCDNA3.1(+)::G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these plasmids occurs at the first gly-ser in the linker [ser-gly-gly-ser-gly-gly-ser] so the recombinants will contain the 4 amino acid (ser-gly-gly-ser) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II piece should be verified.

Example5

Methods to Improve Bioactivities of IgG-C_H Fusion Proteins

All of the IgG-C_H fusion proteins appeared to aggregate during purification and the specific activities of the fused growth factors were reduced ~ 2-3-fold as compared to the analogous IgG-Fc fusions. Aggregation may be due to hydrophobic interactions involving the CH1 domain that normally interfaces with the light chain. Coexpression of IgG light chains with the GF-IgG-C_H fusions should prevent aggregation. We describe three ways to coexpress IgG light and heavy chains below. The experiments described below use IgG-Fc, IgG-C_H, EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA,IgD and IgE antibodies. The DNA sequences of human kappa and lambda light chains are known (Heiter et al., 1980). cDNA sequences encoding the human kappa and / or lambda light chain constant (CL) regions can be obtained by PCR amplification from the human leukocyte single-stranded cDNA (Clontech) or human genomic DNA (Clontech). The DNA sequences of the cloned CL domains should be confirmed prior to use in the experiments described below. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

1. Co-expression of the light chain constant region. A Kozak sequence (Kozak, 1991) and a secretion signal can be added to the 5' end of the light chain constant region to enhance translational initiation and direct the secretion of the light chain constant region. A translational stop codon should be added to the 3' end of the sequence. Appropriate cloning sites can be added to the 5' and 3' ends to allow cloning into the mammalian cell expression vector pREP4 (Invitrogen) under control of the RSV promoter and preceding the SV40 derived polyA addition site. This construct can be used to cotransfect COS cells along with pCDNA3.1(+) derivatives that express, for example, EPO-IgG-C_H and G-CSF-IgG-C_H. Alternatively, both light and heavy chains can be expressed from a single plasmid construct. In this case, the light chain sequence and the flanking promoter and polyA sites from pREP4 should be excised with appropriate restriction enzymes and cloned into pCDNA3.1(+). The EPO-IgG-C_H and G-CSF-IgG-C_H coding sequences could then cloned into the pCDNA3.1(+) polylinker under control of the CMV promoter.

2. Co-expression of a GF-light chain constant region fusion proteins. An alternative mode of light chain expression would be to modify the 5' end to add a portion of a flexible peptide linker sequence fused to the amino-terminus of the CL coding sequence and add a translational stop codon to the 3' end of the sequence. Appropriate cloning sites can be added as well to the 5' and 3' ends to allow cloning as an in frame fusion to the EPO and G-CSF genes cloned in the plasmids pCDNA3.1(+)::EPOfus and pCDNA3.1(+)::G-CSFfus. This plasmid can be cotransfected into COS cells with plasmids that express, for example, EPO-IgG1-C_H and G-CSF-IgG1-C_H. In this instance both heavy and light chains will contain growth factor fusions. The light and heavy chains also could be expressed from a single pCDNA3.1 (+) construct as described above.

3. Light chain-heavy chain fusions. A third mode of "co-expression" would be to modify the 5' and 3' ends of the CL coding sequence to incorporate portions of a flexible peptide linker at both ends. By also incorporating appropriate cloning sites (*Bsp* EI and *Bam* HI) such a construct can be inserted into the Bsp EI and *Bam* HI sites within the flexible peptide linkers of the EPO-IgG-C_H and G-CSF-IgG-C_H fusions in pCDNA3.1(+). The resulting constructs would encode, for example, single polypeptide [EPO]-[CL]-[IgG-C_H] and [G-CSF]-[CL]-[IgG-C_H] fusions. The fusion of the carboxy-terminus of the light chain constant region to the amino-terminus of the heavy chain CH1 domain would be analogous to single chain Fv polypeptides. Flexible peptide linkers of the (ser-gly-gly) motif on the order 14 to 20 residues in length have been used to fuse the carboxy-terminus of the light chain variable region to the amino-terminus of the heavy chain variable domain (Stewart et al., 1995) and could be used to join the CL domain to the IgG-C_H domain.

Example 6

GF-IgG Fusion Proteins with Reduced Complement Binding and Fc Receptor Binding Properties

Certain GF-IgG1 fusion proteins may be toxic or lack efficacy in the animal models due to activation of complement or immune processes related to Fc receptor binding. For this reason, GF-IgG4 fusion proteins may be preferred because IgG4 is less efficient at complement activation and Fc receptor binding than is IgG1 (Roit et al., 1989). The EPO- and G-CSF-IgG4-Fc fusion proteins are as potent or nearly as potent as the IgG1-Fc fusion proteins in *in vitro* bioasays. Alternatively, one can perform *in vitro* mutagenesis experiments, as detailed below, to change specific amino acids in the IgG domains known to be responsible for complement

activation and Fc receptor binding. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in Examples 2 and 3.

A. Complement Binding. Amino acids in IgGs that play a role in complement activation have been localized to the IgG CH2 domain. Specifically, amino acids Glu318, Lys320, Lys322, Ala330 and Pro331 in human IgG1 have been implicated as contributing to complement activation (Isaacs et al., 1998). Substitution of Glu318, Lys320 and Lys322 in IgG1 with alanine residues results in IgG proteins possessing reduced ability to activate complement (Isaacs et al., 1998). The amino acid sequence of IgG4 is identical to IgG1 in this region, yet IgG4 does not activate complement. As an alternative to using IgG4, one can change Glu318, Lys320 and Lys 322 (alone or in combination) of IgGs that have these residues to alanine residues or other amino acids that reduce complement activiation using the PCR-based mutagenesis strategies described in Example 1.

B. Fc Receptor Binding. Human IgG subclasses differ in their ability to bind Fc receptors and stimulate antibody-dependent cell-mediated cytotoxity (ADCC). IgG1, IgG3 and IgG4 are best at stimulating ADCC, whereas IgG2 has significantly reduced ability to stimulate ADCC (Roit et al., 1989). ADCC occurs through a mechanism that involves binding of the antibody to Fc receptors on immune cells. Amino acids responsible for Fc receptor have been localized to the CH2 domain of the IgG molecule. Specifically, amino acids 233-235 have been implicated in Fc receptor binding. Human IgG1 has the amino acid sequence GluLeuLeu in this region, whereas IgG2, which does not bind Fc receptors, has the sequence ProAlaVal. IgG4 has the sequence GluPheLeu in this region and is 10-fold less efficient at binding Fc receptors than IgG1. Substitution of the IgG2 sequence ProAlaVal for GluLeuLeu at positions 233-235 in IgG1or

IgG4 results in IgG1 and IgG4 antibodies with significantly reduced capacity for Fc receptor binding and ADCC (Isaacs et al., 1998). One can introduce these amino acid changes into the GF-IgG fusion protein constructs using the PCR-based mutagenesis strategy described in Example 1. Alternatively one can construct modified GF-IgG fusion proteins in which glycosylation of asparagine 297 in the IgG1 CH2 domain (or the equivalent asparagine residue in the other IgG subclasses) is prevented. Aglycosylated IgG1 antibodies display significantly reduced binding to Fc receptors and ability to lyse target cells as compared to glycosylated IgG1antibodies (Isaacs et al., 1998). One can construct aglycosylated versions of the GF-IgG fusion proteins by changing asparagine-297 to glutamine or another amino acid, or by changing threonine-299, which is part of the glycosylation recognition sequence (N-X-S/T), to alanine or to an amino acid other than serine. The amino acid in the X position of the glycosylation recognition sequence, i.e, amino acid 298, also could be changed to proline to prevent glycosylation of asparagine 297 in the IgG CH2 domain.

Example 7

Pharmacokinetic Experiments with GF-IgG Fusion Proteins

Pharmacokinetic experiments can be performed to demonstrate that the GF-IgG fusion proteins have longer circulating half-lives than the corresponding non-fused proteins. Both intravenous and subcutaneous pharmacokinetic data can be obtained. Terminal pharmacokinetic parameters can be calculated from the intravenous delivery data.

For the intravenous delivery studies, rats (~350g) should receive an intravenous bolus injection (0.1 mg/kg) of the IgG1-Fc fusion protein (EPO or G-CSF) or the corresponding non-fused protein (EPO or G-CSF) and circulating levels of the proteins measured over the course of 144 h. Three rats should be used for each protein sample. Blood samples should be drawn at 0,

0.08, 0.5, 1.5, 4, 8, 12, 24, 48, 72, 96, 120, and 144 h following intravenous administration. Serum levels of the test proteins can be quantitated using commercially available EPO and G-CSF ELISA kits (R & D Systems). Serial dilutions of each blood sample can be analyzed initially in the *in vitro* bioassays to identify dilutions that will fall within the linear range of the ELISAs. (0.025 to 1.6 ng/ml for EPO and 0.04 to 2.5 ng/ml for G-CSF). Titration experiments should be performed to determine the relative sensitivities of the ELISAs for detecting the IgG1-Fc fusion proteins and the corresponding non-fused proteins. This experiment will require 105 μg of each protein.

The subcutaneous delivery studies should follow the same protocol as the intravenous studies except for the route of delivery. Serum levels of the test proteins can be quantitated by ELISA as described above. This experiment will require 105 μg of each protein.

Example 8

Animal Efficacy Models

I*n vivo* efficacy of the EPO-IgG1-Fc and G-CSF-IgG1-Fc fusion proteins can be demonstrated in normal rats and mice. These studies should use a variety of doses and dosing schedules to identify the proper doses and dosing schedules. Efficacy of the GF-IgG fusion proteins also can be demonstrated in appropriate disease models – anemia for EPO-IgG1-Fc and neutropenia for G-CSF-IgG1-Fc. The pharmacokinetic experiments will provide guidance in deciding dosing schedules for the IgG1 fusion proteins to be used for the animal studies. From published results with other IgG-Fc fusion proteins (Richter et al., 1999; Zeng et al., 1995) the GF-IgG fusion proteins should be effective when administered every other day or every third day and possibly less often, e.g. a single injection. The dosing schedules may have to be modified

depending upon the results of the pharmacokinetic studies and initial animal efficacy results. The dose of protein administered per injection to the rodents also may have to be modified based upon the results of the pharmacokinetic experiments and initial animal efficacy results

A. EPO Animal Efficacy Models

In vivo efficacy of the EPO-IgG1-Fc fusion protein (and other EPO-IgG fusion proteins) can be determined in normal rodents. Wild type EPO is known to stimulate increases in hematocrit and erythropoiesis in normal rats or mice. The experiments described below uses rats. Sprague-Dawley rats (~200g) can be purchased from a commercial supplier such as Charles River (Wilmington, MA). Previous studies have shown that administration of 100 IU/kg (approximately 800 ng/kg) of rEPO once per day (160 ng SID / 200 g rat) by subcutaneous injection gives a significant increase in hematocrit and erythropoiesis in rodents (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998; Sykowski et al., 1998). Groups of 5 rats should receive subcutaneous injections of rEPO, EPO-IgG1-Fc or placebo (vehicle solution) at specified intervals for up to five days. A dose equivalent to a molar ratio of EPO (400 ng/200g rat of the EPO-IgG fusion protein) should be tested. Higher or lower doses also should be tested. A wide range of EPO-IgG1-Fc doses (over 500-fold variation) should be tested in these initial experiments to increase the likelihood that one of the doses will be effective. It is possible that administration of too much EPO-IgG1-Fc will impede erythropoiesis due to toxicity. Control rats should receive vehicle solution only. Additional control groups should receive rEPO (160 ng/SID for 5 days) and 160 ng rEPO using the same dosing regimen as EPO-IgG1-Fc. On day 6 the animals should be sacrificed and blood samples collected for hematocrit and complete blood cell count (CBC) analysis. Hematopoietic tissues (liver and spleen) should be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased

erythropoiesis. Bone marrow should be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased erythropoiesis. Comparisons between groups can be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P< 0.05$ should be considered significant.

Daily injections of rEPO should stimulate increases in hematocrit and erythropoiesis in the rats, whereas less frequent administration of the same dose of rEPO should not, or do so to a lesser extent. Dose-dependent increases in these parameters should be observed in the EPO-IgG-Fc-treated animals. Greater increases in these parameters may be observed in the EPO-IgG1-Fc-treated animals than in animals treated with EPO using the less frequent dosing schedules. Significantly less EPO-IgG1-Fc may be required to achieve the same increases in these parameters obtained with daily injections of EPO.

Additional experiments with less frequent dosing, e.g., a single injection, could be performed.

1. EPO Experiment 1 – Normal Rats – Every Other Day Dosing: Rats should receive injections every other day (EOD), i.e. on days 1, 3 and 5, for a total of three injections.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (EOD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng EOD)	5	2.4 µg
4	EPO-IgG1-Fc (0.64 ng EOD)	5	0.0096 µg
5	EPO-IgG1-Fc (3.2 ng EOD)	5	0.048 µg

6	EPO-IgG1-Fc (16 ng EOD)	5	0.24 µg
7	EPO-IgG1-Fc (80 ng EOD)	5	1.2 µg
8	EPO-IgG1-Fc (400 ng EOD)	5	6.0 µg

2. EPO Experiment 2 – Normal Rats – Every Third Day Dosing: Rats should receive injections every third day (ETD), i.e., on days 1 and 4, for a total of two injections.

Group	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	Vehicle solution (ETD)	5	0
2	EPO (160 ng SID)	5	4.0 µg
3	EPO (160 ng ETD)	5	1.6 µg
4	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0064µg
5	EPO-IgG1-Fc (3.2 ng ETD)	5	0.032 µg
6	EPO-IgG1-Fc (16 ng ETD)	5	0.16 µg
7	EPO-IgG1-Fc (80 ng ETD)	5	0.8 µg
8	EPO-IgG1-Fc (400 ng ETD)	5	4.0 µg

3. EPO Experiment 3 - Rat Anemia Model

Cisplatin-induced anemia is a well-characterized rodent model of chemotherapy-induced anemia and has direct relevance to the human clinical setting. rEPO reverses the anemia in this model when administered at daily doses of 100 Units/kg (Matsumoto et al., 1990; Vaziri et al., 1994; Baldwin et al., 1998). EPO-IgG-Fc should be effective at reversing anemia in this model. The dosing schedule for EPO-IgG-Fc to be used in this experiment culd be the one that worked best in the normal rat experiments. The experimental protocol outlined below assumes EPO-IgG-Fc is effective when administered ETD, but this can be altered based upon results of experiments with normal rats. Sprague-Dawley rats (~200g) should be treated on day 0 with an intraperitoneal injection of Cisplatin (3.5mg/kg) to induce anemia and randomized to various treatment groups. The dosing schedule and amounts of protein injected per rat should be as described for the ETD normal rat experiments described above. Rats should receive injections of EPO-IgG-Fc, rEPO or saline on days 1, 4 and 7, for a total of three injections. One control group of rats should receive daily subcutaneous injections of rEPO (100 Units/kg). Another control group should not receive the initial Cisplatin injection but should receive ETD injections of saline. On day 9 the rats should be sacrificed and blood and tissue samples obtained for comprehensive CBC and histopathology analyses.

Group	Cisplatin Treatment	Sample (Dose and Frequency)	Number of Rats	Protein Required
1	-	Vehicle solution (ETD)	5	-
2	+	Vehicle solution (ETD)	5	-
3	+	EPO (160 ng SID)	5	6.4 µg
4	+	EPO (160 ng ETD)	5	2.4 µg
5	+	EPO-IgG1-Fc (0.64 ng ETD)	5	0.0096µg
6	+	EPO-IgG1-Fc (3.2 ng ETD)	5	0.048 µg
7	+	EPO-IgG1-Fc (16 ng ETD)	5	0.24 µg
8	+	EPO-IgG1-Fc (80 ng ETD)	5	1.2 µg
9	+	EPO-IgG1-Fc (400 ng ETD)	5	6.0 µg

B. G-CSF Animal Efficacy Models

The G-CSF-IgG1-Fc experiments can be modeled after the EPO experiments described above. Mice or rats can be used for these experiments. The experiments described below uses mice. One can extrapolate pharmacokinetic data from the rat to the mouse because protein clearance is proportional to body weight (Mahmood, 1998). One can demonstrate efficacy of G-CSF-IgG1-Fc in normal animals using EOD or ETD dosing schedules. G-CSF has been shown to stimulate neutrophil levels in normal and neutropenic rodents at a dose of 100 µg/kg (Kubota et al., 1990; Kang et al., 1995), which is the standard dose that should be used for the experiments. Effectiveness of G-CSF-IgG1-Fc in a mouse neutropenia model can be

demonstrated using the optimum dosing schedule determined in the normal mouse experiments. Protein amounts required assume 20g mice are used.

Groups of 5 mice should receive subcutaneous injections of rG-CSF, G-CSF-IgG1-Fc or placebo (vehicle solution) at specified intervals for up to five days. One of the doses of G-CSF-IgG1-Fc should be a molar equivalent of rG-CSF. A wide range of G-CSF-IgG1-Fc doses (500--fold variation) should be used for these initial experiments to increase the likelihood that one of the doses will be effective. It is possible that administration of too much G-CSF-IgG1-Fc will impede granulopoiesis due to toxicity. Control rats should receive vehicle solution only. Additional control groups should receive rG-CSF (2 μg/SID for 5 days) and 2μg rG-CSF using the same dosing regimen as G-CSF-IgG1-Fc. On day 6 the animals should be sacrificed and blood samples collected for CBC analysis. Hematopoietic tissues (liver and spleen) should be collected, weighed and fixed in formalin for histopathologic analyses to look for evidence of increased granulopoiesis. Bone marrow should be removed from various long bones and the sternum for unit particle preps and histopathologic analysis to look for evidence of increased granulopoiesis. Comparisons between groups should be made using a Students T test for single comparisons and one-way analysis of variance for multiple comparisons. $P< 0.05$ should be considered significant.

Daily injections of rG-CSF should stimulate increases in circulating neutrophils and granulopoieis in the mice, whereas less frequent administration of the same dose of rG-CSF should not, or should do so to a lesser extent. Dose-dependent increases in these parameters should be observed in the G-CSF-IgG1-Fc-treated animals. Greater increases in these parameters may be observed in the G-CSF-IgG1-Fc-treated animals than in animals treated with rG-CSF using the less frequent dosing schedules. Significantly less G-CSF-IgG1-Fc may give the same increases in these parameters obtained with daily injections of rG-CSF.

1. G-CSF Experiment 1 – Normal Mice: Mice (~20g) should receive injections every other day (EOD), i.e. on days 1, 3 and 5, for a total of three injections. On day 6 the animals should be sacrificed and analyzed.

Group	Sample (Dose and Frequency)	Number of Mice	Protein Required
1	Vehicle solution (EOD)	5	0
2	G-CSF (2 μg SID)	5	50 μg
3	G-CSF (2 μg EOD)	5	30 μg
4	G-CSF-IgG1-Fc (0.008 μg EOD)	5	0.12 μg
5	G-CSF-IgG1-Fc (0.04 μg EOD)	5	0.6 μg
6	G-CSF-IgG1-Fc (0.2 μg EOD)	5	3 μg
7	G-CSF-IgG1-Fc (1 μg EOD)	5	15 μg
8	G-CSF-IgG1-Fc (5 μg EOD)	5	75 μg

2. G-CSF Experiment 2 – Normal Mice – Every Third Day Injection: Mice (~20g) should receive injections every third day (ETD), i.e., on days1 and 4, for a total of two injections.

Group	Sample (Dose and Frequency)	Number of Mice	Protein Required
1	Vehicle solution (ETD)	5	0
2	G-CSF (2 μg SID)	5	50 μg
3	G-CSF (2 μg ETD)	5	20 μg
4	G-CSF-IgG1-Fc (0.008 μg ETD)	5	008 μg
5	G-CSF-IgG1-Fc (0.04 μg ETD)	5	0.4 μg
6	G-CSF-IgG1-Fc (0.2 μg ETD)	5	2 μg
7	G-CSF-IgG1-Fc (1 μg ETD)	5	10 μg
8	G-CSF-IgG1-Fc (5 μg ETD)	5	50 μg

Additional experiments with even less frequent dosing, e.g., a single injection, can be performed.

3. G-CSF Experiment 3 –Neutropenic Mice: Efficacy of G-CSF-IgG1-Fc also can be demonstrated in neutropenic animals. Neutropenia can be induced by treatment with cyclophosphamide (CPA; 100 mg/kg), which is a commonly used chemotherapeutic agent that is myelosuppressive and relevant to the human clinical setting. G-CSF accelerates recovery of normal neutrophil levels in cyclophosphamide-treated animals (Kubota et al., 1990; Kang et al., 1995). Mice (~20g) should receive an intraperitoneal injection of cyclophosphamide on day 0 to

induce neutropenia. The animals should be divided into different groups, which will receive subcutaneous injections of G-CSF, G-CSF-IgG1-Fc or placebo. One control group should not receive cyclophosphamide but should receive placebo injections. The experiment described below assumes G-CSF-IgG1-Fc will be effective when administered ETD and that this is the dosing schedule that will be used for this experiment. The exact dosing schedule to be used will be determined by the results of the pharmacokinetic experiments and normal mouse efficacy studies described above. Mice should receive injections of test substances every third day (ETD), i.e., on days 1 and 4 for a total of two injections. On day six the animals should be sacrificed and blood and tissue samples analyzed as described above.

Group	CPA	Sample (Dose and Frequency)	Number of Mice	Protein Required
1	-	Vehicle solution (ETD)	5	0
2	+	Vehicle solution (ETD)	5	0
3	+	G-CSF (2μg SID)	5	50 μg
4	+	G-CSF (2 μg ETD)	5	20 μg
5	+	G-CSF-IgG1-Fc (0.008 μg ETD)	5	0.08 μg
6	+	G-CSF-IgG1-Fc (0.04 μg ETD)	5	0.4 μg
7	+	G-CSF-IgG1-Fc (0.2 μg ETD)	5	2 μg
8	+	G-CSF-IgG1-Fc (1 μg ETD)	5	10 μg
9	+	G-CSF-IgG1-Fc (5 μg ETD)	5	50 μg

Example 9

Separating IgG Fusion Protein Monomers from Dimers

The final purification scheme for the GF-IgG fusion proteins could include additional column chromatography steps in addition to affinity chromatography to remove protein contaminants. For use as human therapeutics it will be preferable to obtain preparations of GF-IgG monomers substantially free from GF-IgG dimers, and preparations of GF-IgG dimers substantially free from GF-IgG monomers. GF-IgG dimers can be separated from GF-IgG monomers using a variety of column chromatography procedures known to those with skill in the art. Examples of such procedures include ion-exchange, size exclusion, hydrophobic interaction, reversed phase, metal chelation, affinity columns, lectin affinity, hydroxy apatite and immobilized dye affinity chromatography. Other useful separation procedures known to those skilled in the art include salt precipitation, solvent precipitation/extraction and polyethylene glycol precipitation. Endotoxin levels in the purified proteins should be tested using commercially available kits to ensure that they are not pyrogenic.

Example 10

The procedures described in the preceding examples can, with minor modifications, be used to created IgG fusions with other proteins. Examples of other IgG fusion proteins that would find therapeutic uses in humans include IgG fusions of interferons alpha, beta and gamma, IL-11, TPO and GM-CSF. DNAs encoding these proteins can be cloned as described below and fused to the various IgG domains described in Example 1. The recombinant fusion proteins can be expressed and purified as described in Example 2. The purified fusion proteins can be tested in appropriate *in vitro* bioassays to determine their specific activities. DNA sequences, encoded amino acids and appropriate *in vitro* and *in vivo* bioassays for these proteins are well known in

the art and are described in Aggarwal and Gutterman (1992; 1996), Aggarwal (1998), and Silvennoimem and Ihle (1996). Bioassays for these proteins also are provided in catalogues of commercial suppliers of these proteins such as R&D Systems, Inc, Endogen, Inc., and Gibco BRL.

1.Cloning human alpha interferon. Alpha interferon is produced by leukocytes and has antiviral, anti-tumor and immunomodulatory effects. There are at least 20 distinct alpha interferon genes that encode proteins that share 70% or greater amino acid identity. Amino acid sequences of the known alpha interferon species is given in Blatt et al., 1996). A "consensus" interferon that incorporates the most common amino acids into a single polypeptide chain has been described (Blatt et al, 1996). A hybrid alpha interferon protein may be produced by splicing different parts of alpha interferon proteins into a single protein (Horisberger and Di Marco, 1995). The following example describes construction of an alpha 2 interferon IgG fusion protein. Similar procedures can be used to create IgG fusions of other alpha interferon proteins.

DNA encoding human alpha interferon (IFN-α2) was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with BB93 (5>CGCGAATTCGGATATGTAAATAGATACACAGTG>3) and BB94 (5>CGCAAGCTTAAAAGATTTAAATCGTGTCATGGT>3) BB93 anneals to genomic sequences ~300 bp upstream (i.e. 5' to) of the IFN-alpha2 coding sequence and contains an *Eco* RI site for cloning purposes. BB94 anneals to genomic sequences ~100 bp downstream (i.e. 3' to) of the IFN-alpha2 coding sequence and contains a *Hind* III site for cloning purposes. The PCR reaction employed 1X PCR reaction buffer (Promega Corp., Madison WI), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each oligonucleotide primer, 0.33 μg of genomic DNA and 0.4 units of Taq polymerase (Promega) in a 33μl reaction. The reaction consisted of 96°C for 3 minutes

followed by 35 cycles of: [95°C for 60 sec., 58°C for 75 sec., 72°C for 90 sec.] followed by chilling the sample to 6°C. Reactions were carried out in a "Robocycler" thermal cycler (Stratagene Inc. , San Diego, CA). The resulting ~ 1 kb PCR product was digested with *Eco* RI and *Hind* III and cloned into similarly digested, and alkaline phosphatased, pCDNA3.1(+) (Invitrogen, San Diego, CA). A clone having the correct DNA sequence for IFN-α2 (Henco et al, 1985) was identified and designated pBBT160.

In order to construct and express gene fusions of IFN-α2 with IgG coding sequences the IFN-α2 gene was modified at the 5' and 3' ends using PCR based mutagenesis. PBBT160 plasmid DNA was used as template for PCR with primers BB108 and BB109. PCR reactions were carried out with forward primer BB108 (5' CGCAAGCTTGCCACCATGGCCTTGACCTTT GCTTTA-3') and reverse primer BB109 (5'-CGCGGATCCTCCGGATTCCTTACTT CTTAAACTTTC-3'). Primer BB108 anneals to the 5' end of the coding sequence for the IFN-α2 secretion signal and the reverse primer, BB109, anneals to the 3' end of the IFN-α2 coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Henco et al., 1985) was designated pCDNA3.1(+)::IFNAfus or pBBT190 .

2. Cloning human beta interferon. Beta interferon is producedby fibroblasts and exhibits antiviral, antitumor and immunoodulatory effects. Beta interferon is the product of a single gene. DNA encoding human beta interferon (IFN-β) was amplified by PCR from human genomic DNA (CLONTECH). PCR reactions were carried out with forward primer BB110 (5'-

CGCAAGCTTGCCACCATGACCAACAAGTGTCTCCTC-3') and reverse primer BB111 (5'-CGCGGATCCTCCGGAGTTTCGGAGGTAACCTGTAAG-3'). Primer BB110 anneals to the 5' end of the coding sequence for the IFN-β secretion signal and the reverse primer, BB111, anneals to the 3' end of the IFN-β coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Derynck et al., 1980) was designated pCDNA3.1(+)::IFNBfus or pBBT191.

3. Cloning human gamma interferon. Gamma interferon is produced by activated T cells and exhbits anti-viral, antitumor and immunomodulatory effects. A cDNA encoding human gamma interferon (IFN-γ) was amplified by PCR from total RNA isolated from the human Jurkat T cell line (available from the American Type Culture Collection, Rockville, MD). The cells were grown in RPMI media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were activated *in vitro* for 6 hours with 1 μg/ml PHA-L (Sigma chemical Company, catalogue L-4144) and 50 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma Chemical Company, catalogue # P-1585) to induce IFN-γ expression prior to RNA isolation (Weiss et al., 1984; Wiskocil et al., 1985). RNA was isolated from the cells using an RNeasy Mini RNA isolation kit purchased from Qiagen, Inc. (Santa Clarita, CA) following the manufacturer's directions. Approximately 104 μg of total RNA was isolated from 2.4×10^7 cells. First strand synthesis of single-stranded cDNA was accomplished using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) from Boehringer Mannheim Corp (Indianapolis, IN) and random hexamers were used as the primer. Subsequent PCR reactions using the products of

the first strand synthesis as template were carried out with forward primer BB112 (5'-CGCAAGCTTGCCACCATGAAATATACAAGTTATATC-3') and reverse primer BB113 (5'-CGCGGATCCTCCGGACTGGGATGCTCTTCGACCTTG-3'). Primer BB112 anneals to the 5' end of the coding sequence for the IFN-γ secretion signal and the reverse primer, BB113, anneals to the 3' end of the IFN-γ coding sequence. The resulting PCR product was digested with *Hin*d III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hin*d III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Gray et al., 1982) was designated pCDNA3.1(+)::IFNGfus or pBBT192.

4. Construction of Interferon-IgG fusions. The IgG1-Fc coding sequence was fused to the carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~790 bp *Bam* HI – *Xba* I fragment was excised from plasmid pBBT167 [described above in Example 1] and cloned into pBBT190, pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. Similarly, IgG4-Fc coding sequence also was fused to IFN-α2, IFN-β, and IFN-γ. The ~790 bp *Bam* HI – *Xba* I fragment of plasmid pBBT158 [described above in Example 1] was excised, gel –purified and cloned into pBBT190, pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The IgG1-C_H coding sequence was fused to the carboxyterminus of IFN-α2, IFN-β, and IFN-γ. The ~1080 bp *Bam* HI – *Xba* I fragment of plasmid pBBT166 [described above in Example 1] was excised and cloned into pBBT190, pBBT191 and pBBT192 which had been digested with *Bam* HI and *Xba* I, and treated with alkaline phosphatase. The structures of the resulting recombinant plasmids were

verified by restsriction endonuclease digestions and agarose gel electrophoresis. These plasmids and the IFN-IgG fusion proteins that they encode are listed in Table 5.

Table 5. Interferon – IgG Fusion Proteins

Expression Plasmid	IFN Fusion Protein
PBBT193	IFN-α2-IgG1-Fc
PBBT194	IFN-α2-IgG4-Fc
PBBT220	IFN-α2-IgG1-C_H
PBBT195	IFN-β-IgG1-Fc
PBBT196	IFN-β-IgG4-Fc
PBBT221	IFN-β-IgG1-C_H
PBBT209	IFN-γ-IgG1-Fc
PBBT210	IFN-γ-IgG4-Fc
PBBT222	IFN-γ-IgG1-C_H

5. Bioactivities of Interferon-IgG Fusion Proteins.

In vitro biological assays for interferons include antiviral assays and cell proliferation inhibition assays. Proliferation of the human Daudi cell line (American Type Culture Collection, Rockville, MD) is inhibited by alpha, beta and gamma interferon and can be used to assay these proteins (Horoszewicz et al., 1979; Evinger and Pestka, 1981). Daudi cells are maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. Bioassays are performed in this media using the procedures described above except that the number of cells added to each well of a 96 well plate should be 5-20 x 10^3 and the

plates incubated at 37^{0}C for 3 to 4 days. The Daudi cells should be at early saturation density (1-2 x 10^6 cells/ml) before use in the assays for optimum effectiveness of the interferon. Serial dilutions of recombinant alpha, beta and gamma interferon (Endogen; R&D Systems; GibcoBRL, US Biological) should be analyzed in parallel. Recombinant alpha interferon has an IC_{50} (amount of protein to inhibit proliferation by 50%) of approximately 5-30 pg/ml.

Bioactivities of alpha, beta and gamma interferons also can be measured using viral plaque inhibition assays. These assays measure the ability of the interferon protein to protect cells from viral infection. Methods for performing these assays are described in Ozes et al.,(1992) and Lewis (1987; 1995). Human HeLa or WISH cells (available from the American Type Cuture Collection) should be plated in 96-well plates ($3x10^4$ cells/well) and grown to near confluency at 37^{0}C. The cells should be washed and treated for 24 hour with serial 2-3-fold dilutions of each IFN-IgG fusion protein preparation. Vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) should then be added at a multiplicity of infection of 0.1 and the plates incubated for a further 24-48 hours at 37^{0}C. Additional controls should include samples without virus. When 90% or more of the cells have been killed in the virus-treated, no IFN control wells (determined by visual inspection of the wells), the cell monolayer should be stained with crystal violet (0.5% in 20% methanol) and absorbance of the wells read using a microplate reader. Alternatively, 20 µl of MTS/PMS mixture (CellTiter 96 AQueous One Solution, Promega Corporation, Madison, WI) can be added to the cell monolayers and absorbance the wells read at 490 nm after 1-4 hours later as described in Example 3. EC_{50} values (the amount of protein required to inhibit the cytopathic effect of the virus by 50%) should be used to compare the relative potencies of the fusion proteins and non-fused wild type proteins. Wild type IFN proteins protect cells from the cytopathic effects of VSV and EMCV and have

specific activities of approximately $1x10^7$ to $2x10^8$ units/mg in this assay, depending upon the IFN species (Ozes et al., 1992).

The IFN-α-IgG and IFN-β-IgG fusion proteins listed in Table 5 were expressed in COS cells and purified as described in Examples 1 and 2. Non-reducing SDS-PAGE analysis showed that the IFN-α-IgG1-Fc and IFN-α-IgG1-C_H fusion proteins consisted predominantly of disulfide-linked dimers; however small amounts of monomeric fusion protein was observed in all of the samples. The IFN-α-IgG1-C_H fusion protein also contained a significant amount of disulfide-linked aggregates, which failed to enter the gel. The IFN-α-IgG4-Fc fusion protein also was predominantly dimeric; more monomer was present in this sample than in the IFN-α-IgG1-Fc samples. The IFN-β-IgG1-Fc and IFN-β-IgG1-C_H fusion proteins also were largely dimeric, with small amounts of monomeric fusion protein present in each sample. In contrast, the majority of the purified IFN-β-IgG4-Fc fusion protein was monomeric; the remainder was dimeric. Significant amounts of disulfide-linked aggregates were present in all of the purified IFN-β-IgG fusion proteins.

The purified IFN-α-IgG and IFN-β-IgG fusion proteins were assayed using the Daudi cell growth inhibition assay described above. All of the IFN-α-IgG and IFN-β-IgG fusion proteins were biologically active. IC_{50} values for each protein were calculated and are shown in Table 6. Control recombinant IFN-α and IFN-β were purchased from Endogen, Inc. (Woburn, MA) and US Biological (Swampscott, MA), respectively.

Table 6. Bioactivities of IFN-α-IgG and IFN-β-IgG Fusion Proteins

Clone	Protein	IC_{50} Range (ng/ml) [1]	Mean IC_{50} (ng/ml)
-	rhIFN-α	0.015, 0.010	0.013
PBBT193	IFN-α2-IgG1-Fc	1.8, 2.5	2.1
PBBT194	IFN-α2-IgG4-Fc	2.5, 3.5	3.0
PBBT220	IFN-α2-IgG1-C_H	3.5	3.5
-	rhIFN-β	0.18, 0.3	0.24
PBBT195	IFN-β-IgG1-Fc	175, 200	188
PBBT196	IFN-β-IgG4-Fc	15, 15	15
PBBT221	IFN-β-IgG1-C_H	90	90

[1] Data from individual assays

6. Cloning IL-11. IL-11 stimulates evelopment of megakaryocyte precursors of platelets. A cDNA encoding human IL-11 can be amplified by PCR from RNA isolated from human cell lines that express IL-11 such as the human bladder carcinoma cell line 5367 and the HL60 and U937 leukemia cell lines (available from the American Type Culture Collection). PCR reactions can be carried out with forward primer IL-11F (5'-CGCAAGCTTGCCACCATGAACTGTGTTTGCCGCCTG–3') and reverse primer IL-11R (5'-CGCGGATCCTCCGGACAGCCGAGTCTTCAGCAGCAG–3'). Primer IL-11F anneals to the 5' end of the coding sequence for the IL-11 secretion signal and the reverse primer, IL-11R,

anneals to the 3' end of the IL-11 coding sequence. The resulting PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones should be sequenced to identify one with the correct DNA sequence. The human cell line TF-1 (available from the American Type Culture Collection) can be used to measure bioactivities of the IL-11-IgG fusion proteins.

7. Cloning TPO. Thrombopoietin (TPO) stimulates development of megakaryocyte precursors of platelets. A cDNA encoding human TPO can be amplified by PCR from single-stranded cDNA prepared from liver or kidney, which is available from commercial sources such as CLONTECH and Stratagene, Inc.. PCR reactions can be carried out with forward primer TPOF (5'- CGCAAGCTTGCCACCATGGAGCTGACTGAATTGCTC –3') and reverse primer TPOR (5'- CGCGGATCCTCCGGACCCTTCCTGAGACAGATTCTG –3'). Primer TPOF anneals to the 5' end of the coding sequence for the TPO secretion signal and the reverse primer, TPOR, anneals to the 3' end of the TPO coding sequence. The resulting PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones should be sequenced to identify one with the correct DNA sequence. The cell line MO7e (Avanzi et al., 1988) can be used to measure TPO bioactivity.

8. Cloning GM-CSF: A cDNA encoding human GM-CSF can be amplified by PCR from RNA isolated from a cell line that expresses GM-CSF such as the human T cell line HUT 102 (available from American Type Culture Collection) or from human peripheral blood lymphocytes or the human Jurkat T cell line that had been activated with 20 μg/ml concanavalin

A (Sigma Chemical Company) and 40 ng/ml phorbol myristate acetate (PMA, Sigma Chemical Company). PCR reactions can be carried out with forward primer GMCSFF (5'-CGCAAGCTTGCCACCATGTGGCTGCAGAGCCTGCTG-3') and reverse primer GMCSFR (5'- CGCGGATCCTCCGGACTCCTGGACTGGCTCCCAGCA 3'). Primer GMCSFF anneals to the 5' end of the coding sequence for the GM-CSF secretion signal and the reverse primer, GMCSFR, anneals to the 3' end of the GM-CSF coding sequence. The resulting PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones should be sequenced to identify one with the correct DNA sequence . Bioactivity of the GM-CSF-IgG fusion proteins can be measured using the TF-1 cell line (available from the American Type Culture Collection).

Example 11

Multimeric Cytokine and Growth Factor fusion Proteins

Bioactive fusion proteins also can be created by constructing multimeric fusion proteins of the growth factors and cytokines mentioned in this application. These multimeric fusion proteins can be constructed as described for the IgG fusion proteins except that a second growth factor/cytokine protein can be substituted for the IgG domain. The two growth factors/cytokines can be joined together with or without linker amino acids between the two growth factors/cytokines. Suitable peptide linkers include those described in Examples 1 and 4. The fusion proteins can be homodimeric, heterodimeric, homomultimeric (comprising three or more copies of the same growth factor/cytokine) or heteromultimeric (comprising two or more different growth factors/cytokines). The most carboxy-terminal cytokine/growth factor domain should be modified using procedures such as PCR to delete the protein's natural signal sequence

and add, if desired, a short peptide linker sequence preceding the first amino acid of the mature protein sequence. The linker sequence could include a restriction enzyme site to facilitate joining to the amino-terminal cytokine/growth factor domain. In multimeric fusion proteins the cytokine/growth factor domains not at the amino- or carboxy-terminus of the protein can be modified to delete the natural signal sequence and termination codon and add, if desired, peptide linkers to the amino- and carboxy-termini of the protein. The fusion proteins can expressed in COS cells following transfection, purified and tested in appropriate *in vitro* bioassays.

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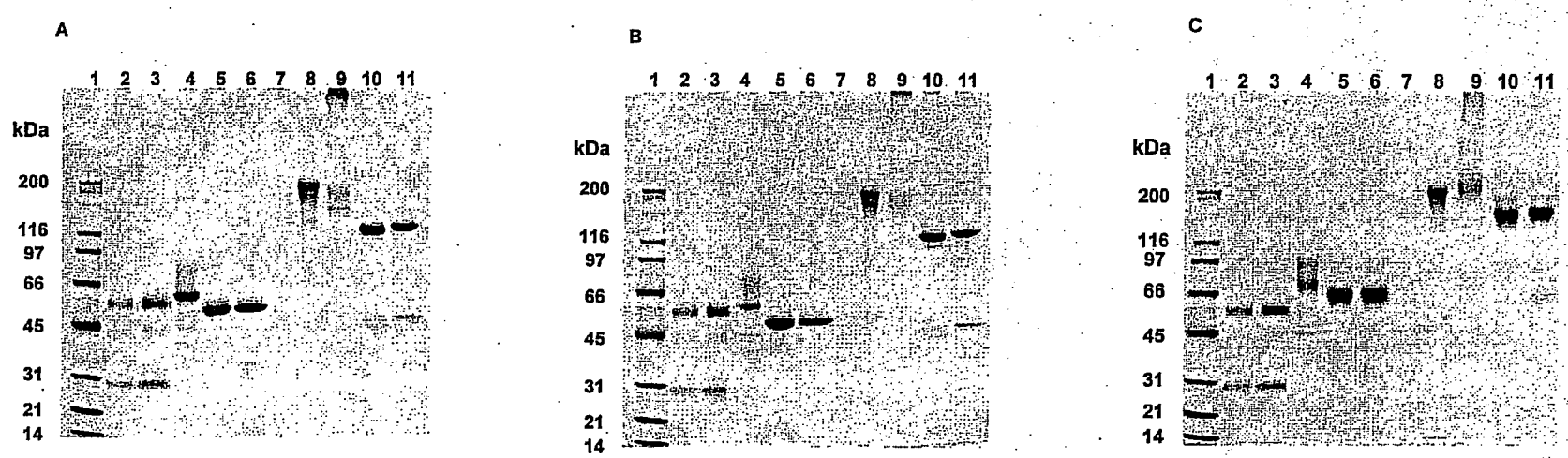
Figure 1.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